FINAL

Report on Carcinogens Background Document for

Human Papillomaviruses: Genital-Mucosal Types

June 5, 2003

Prepared for the:
U.S. Department of Health and Human Services
Public Health Service
National Toxicology Program
Research Triangle Park, NC 27709

Prepared by:
Technology Planning and Management Corporation
Canterbury Hall, Suite 310
4815 Emperor Blvd
Durham, NC 27703
Contract Number N01-ES-85421

FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of all substances (i) that either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens; and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (DHHS) has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP) who prepares the Report with assistance from other Federal health and regulatory agencies and non-government institutions.

Nominations for listing in or delisting from the RoC are reviewed by a formal process that includes a multi-phased, scientific peer review and multiple opportunities for public comment. The review groups evaluate each nomination according to specific RoC listing criteria. This Background Document was prepared to assist in the review of the nomination of High Risk Human Papillomaviruses. The scientific information in this document comes from publicly available, peer-reviewed sources. Any interpretive conclusions, comments or statistical calculations, etc., made by the authors of this document that are not contained in the original citation are identified in brackets [ ]. If any member(s) of the scientific peer review groups feel this Background Document does not adequately capture and present the relevant information they will be asked to write a commentary for this Background Document that will be included as an addendum to the document. In addition, a meeting summary that contains a brief discussion of the respective review group's review and recommendation for the nomination will be added to the Background Document, also as an addendum.

A detailed description of the RoC nomination review process and a list of all nominations under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at http://ntp-server.niehs.nih.gov. The most recent RoC, the 9th Edition, was published in May, 2000 and may be obtained by contacting the NIEHS Environmental Health Information Service (EHIS) at http://ehis.niehs.nih.gov (800-315-3010).

CONTRIBUTORS

NIEHS/NTP Staff

C.W. Jameson, Ph.D.	Head, Report on Carcinogens, Environmental Toxicology Program, NIEHS
Ruth M. Lunn, Dr. P.H.	Report on Carcinogens Group, Environmental Toxicology Program, NIEHS
Shawn Jeter, B.S.	Report on Carcinogens Group, Environmental Toxicology Program, NIEHS
AnnaLee Sabella	Report on Carcinogens Group, Environmental Toxicology Program, NIEHS

Support to the National Toxicology Program for the preparation of this background document was provided by Technology Planning and Management Corporation through NIEHS Contract Number NO1-ES-85421

Ronald Thomas, Ph.D., Principal Investigator

Sanford Garner, Ph.D., Co-Principal Investigator

Stanley Atwood, M.S., DABT

Susan Goldhaber, M.S.

Greg Pazianos, B.S.

Ashlee Duncan, M.S.

Ibrahim Raphiou, Ph.D.

Support staff

Angie Fralick, B.S.

Tracy Saunders, B.S.

Consultants

Karl Munger, Ph.D., Pathology Department and Harvard Center for Cancer Biology, Harvard Medical School, Boston, MA

Keerti Shah, Ph.D., Department of Molecular Microbiology and Immunology Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD

Mark Schiffman, M.D., Chief, Interdisciplinary Studies Section, Environmental Epidemiology Branch, Divison of Cancer, Epidemiology, and Genetics, National Cancer Institute, Bethesda, MD

Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

U.S. Department of Health and Human Services

National Toxicology Program

Known to be Human Carcinogens:

There is sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

Reasonably Anticipated to be Human Carcinogens:

There is limited evidence of carcinogenicity from studies in humans, which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or

There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either a *known to be human carcinogen* or *reasonably anticipated to be human carcinogen* or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

Executive Summary

Introduction

Human papillomaviruses (HPV) are DNA viruses that belong to the papillomaviridae family, which includes more than 100 types specific to humans and additional virus types that infect only specific animal species. The HPVs infect the squamous epithelium of the skin and mucosal membranes. This document is restricted to a review of the genital-mucosal-associated types, which include more than 35 types that have been classified in the literature as either high risk or low risk. High-risk viruses are associated with cervical cancer, whereas low-risk viruses are associated with genital warts or low-grade cervical intraepithelial neoplasia. Most studies to date have considered HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 to be high-risk viruses; some studies also include other HPVs, most notably HPV-66. Classification of viruses is also based on phylogenetic and mechanistic considerations. Most high-risk viruses share homology with HPV-16 and HPV-18. Mechanistic studies have shown that high-risk but not low-risk viruses immortalize human keratinocytes, interact with pRb and p53, and induce chromosomal aberrations. However, most mechanistic studies have evaluated only a limited number of viruses, with the majority of the studies focused on HPV-16 and HPV-18 and a few studies on HPV-31 and HPV-33.

HPVs are small viruses consisting of approximately 8,000 base pairs of covalently closed, double-stranded DNA. The viral genome is divided into a series of open reading frames (ORFs) that encode the relatively few viral-specific proteins and a long control region (LCR) that contains the origin of replication and regulatory elements. The early genes are designated E1 through E8 and are associated with regulation of transcription (e.g., E2) and cellular proliferation (e.g., E6 and E7). The late ORFs (L1 and L2) encode proteins that comprise the viral capsid structure and are produced late in the infectious cycle of the virus. Papillomaviruses specifically target squamous epithelial cells, and productive infection of cells is linked to the differentiation stage of epithelial cells; the early stages occur in the dividing cells of the basal squamous epithelium, whereas the late stages occur in terminally differentiated squamous epithelial cells.

Human exposure

HPV infection is one of the most common sexually transmitted infections, worldwide. More than two million people per year in the United States develop cytologic abnormalities of the genital tract that are caused by HPV infections. The major risk factors for HPV infection are behaviors associated with sexual activity. Thus, the highest prevalence rates (25% to 40%) are found among young, sexually active individuals. Although most infections are asymptomatic and are resolved spontaneously, HPV infection can lead to clinical disease, including anogenital warts, cervical neoplasia, cervical cancer, and other anogenital cancers. Diagnosis of HPV infections is made by either conventional clinical methods (e.g., visible lesions or microscopic cytology) or by molecular diagnosis (e.g., DNA-based methods). Human epidemiological studies typically assess HPV infection by molecular diagnosis (HPV DNA) or serological

responses. Treatment of HPV infections depends on the severity of the disease and includes topical applications, interferon-related therapies, laser methods, and surgical excision. Early-phase clinical trials of prophylactic vaccines are currently being conducted using HPV-16 L1 capsid antigens.

Human cancer studies

IARC

The International Agency for Research on Cancer (IARC) classified HPV-16 and HPV-18 as carcinogenic to humans (Group 1) based on sufficient evidence in humans. HPV-31 and 33 were classified as probably carcinogenic to humans (Group 2A) based on limited evidence in humans, and some other HPV types other than HPV-16, 18, 31 as possibly carcinogenic to humans (Group 2B). IARC also noted that there was evidence suggesting lack of carcinogenicity to the cervix in humans of HPV types 6 and 11.

Current studies

At the time of the 1995 IARC review, there were a limited number of studies evaluating the effects of HPV types other than 16 and 18, and a limited number of cohort and analytical studies on cancer at sites other than the cervix. IARC initiated a series of case-control studies in various geographical locations to evaluate cancer risk from individual HPV types and also to evaluate the role of cofactors in HPV-carcinogenesis. A meta-analysis for the different HPV types using data from these studies has been performed. The findings from current case-control and cohort studies, including the meta-analysis for individual viruses and for classes of viruses are summarized in the table below:

Summary of the evidence for the carcinogenicity of HPV viruses in humans

HPV	**Cervical cancer**		**Other anogenital cancer**
	Case control (relative risk)	**Cohort (relative risk)**	**Seroepidemiological**
HPV-16	Strong evidence *DNA* Cancer: 83 to 910 Meta-analysis: 150 *Seroepidemiological* High-grade CIN and/or cancer: ~ 3.0	Strong evidence *DNA* Cancer or carcinoma *in situ*: 16 to 105 High-grade SIL: 8.9 to 64 Seroepidemiological High-grade SIL and/or cancer: 2.2 to 12.5 Meta-analysis (cancer): 3.3 (95% CI =1.1 to 4.9)	Consistent positive associations with vulvar and vaginal cancer Somewhat weaker associations with penile and anal cancer

HPV	**Cervical cancer**		**Other anogenital cancer**
	Case control (relative risk)	**Cohort (relative risk)**	**Seroepidemiological**
HPV-18	Strong evidence *DNA* Cancer: 93 to 276 (up to 946 for adenocarcinoma) Meta-analysis: 182 *Seroepidemiological* cancer and CIN III: ~ 2 to 3	Moderate to strong evidence *DNA* No cancer studies High-grade SIL or carcinoma *in situ:* ~ 2 to 3 *Seroepidemiological* cancer, carcinoma *in situ*, CIN III: 2 to 3	Some evidence for vulvar and vaginal cancer but inconsistent across studies
Other HPVs: 31, 33, 35, 39, 45, 51, 52, 58, 59, 66	Strong evidence for HPV-31, 33, 35, 45, 51, 52, 58 and 59 *DNA* Cancer Meta-analysis: HPV-31: 60 HPV-33: 78 HPV-35: 35 HPV-45: 151 HPV-51: 43 HPV-52: 146 HPV-58: 79 HPV-59: 347 Other individual studies (one study each): HPV-39: 177 HPV-66: ∞*	Some evidence for HPV-31, 33, 52 and 58 *DNA* High-grade SIL: Elevated but not significant risks for HPV-31, 52 and 58 (1 study) *Seroepidemiological* HPV-33 Cancer: 1.5 CIN: 1.1 to 2.7	Not studied
High-risk HPVs as a class: 16, 18, 31, 33, 39, 45, 51, 52, 5;6, 58, and 68; some studies include 66**	Strong evidence *DNA* High relative risk for HPV-16 and 18 related families, the HPV group (31, 33, 35, 39) and high-risk as a class	Strong evidence *DNA* High-grade CIN: most studies ~ 4.0, 1 study, 31 CIN III: 240 CIN III and cancer: 24 Cancer: 28 Meta-analysis High-grade SIL: 38 Cancer: 17	Not studied

* 66 only detected with other high-risk viruses

** Only DNA studies available

Head and neck cancer

Most of the prospective seroepidemiological or population-based, case-control studies support a role of HPV in head and neck cancer, with the strongest evidence for oropharynx-related tumors. A meta-analysis of case-report and clinical series of oral SCC calculated an odds ratio (OR) of 5.2 for detecting HPV. HPV-16 was the most commonly detected HPV, and other high-risk types were found more frequently than low-risk types.

Cofactors

The International Biological Study of Cervical Cancer detected HPV in 99.7% of more than 1,000 invasive cervical cancer tissues from 22 countries, suggesting that HPV may be necessary for cervical cancer development. Nonetheless, not all individuals positive for HPV infection develop cervical cancer. Most HPV infections are transient and are cleared within 1 to 2 years, and thus confer little risk for cancer development. The actual risk factor appears to be a persistent HPV-16 or other high-risk HPV infection. The factors that cause HPV infections to persist probably depend both on viral characteristics, such as persistence, high-risk strains or variants, and on host cofactors. A higher risk of cervical intraepithelial neoplasia (CIN) and cancer has been observed for viral persistence. Recent studies are beginning to identify these factors and how they interact with HPV, but the exact mechanisms have not been elucidated. Both mechanistic and epidemiological findings of host-cofactors are discussed below.

Smoking

Some, but not all, epidemiological studies suggest that smoking increases the risk of HPV-carcinogenicity. Mutagenic or immunosuppressant components of cigarette smoke may lead to viral persistence, genetic damage, and cancer development. Mechanistic studies have shown that smoking concentrates can transform HPV-16 immortalized endocervical cells.

Hormones

Some, but not all, case-control studies have shown that oral contraceptives and parity increase the risk of HPV-associated cervical cancer. A meta-analysis calculated an OR of 5.0 for at least 10 years' use of oral contraceptives and an OR of 3.8 for seven or greater full-term pregnancies. Mechanistic studies have shown a synergistic relationship between estrogen exposure and HPV-16 oncogenes that resulted in gynecologic pathology in female mice. Other studies have reported that estrogen metabolites promoted anchorage-independent growth in HPV-16 immortalized keratinocytes and that progesterone may increase expression of HPV-16 E6 and E7 transcripts.

Immunity

The host immune system may be the most important cofactor in HPV-carcinogenicity. Most of the evidence comes from studies in the HIV population. HIV-infected individuals have a greater risk for developing HPV-associated cancer, and the risk is greater in HIV patients who are more immunosuppressed, as measured by T-lymphocyte

counts. HIV appears to be a more important risk for persistence of HPV-related SIL than for HPV invasion. There is some evidence to suggest that human leukocyte antigens (HLA) Class I and Class II may modulate HPV-carcinogenic risk.

Conclusion

Numerous case-control studies have consistently demonstrated very strong associations with HPV-16, HPV-18, or high-risk HPVs as a class, and cervical cancer. Moreover, several recent case-control studies have provided strong evidence of positive associations with other individual HPVs, including HPV-31, 33, 35, 39, 45, 51, 52, 58, or 59, and cervical cancer. There is also strong evidence of an association between HPV-16 infection and other anogenital cancers, especially vulvar cancer. Cohort studies have demonstrated that HPV-16 infection occurs before the development of high-grade CIN, CIN III, or invasive cancer. The evidence is weaker for high-risk HPVs as a class and for individual high-risk viruses possibly due to a lower prevalence of these viruses. For the other HPV viruses, the evidence from cohort studies appears to be strongest for HPV-18 and high-risk HPVs as a class. The large ORs observed also suggest that confounding by other agents is unlikely, and many studies have adjusted for most possible confounders. Lastly, the findings of epidemiological studies are supported by mechanistic studies that demonstrate that high-risk HPVs code for oncogenic proteins and can immortalize both cervical and epithelial cells.

Studies in experimental animals

Because of the species specificity of papillomaviruses, laboratory animals cannot be experimentally infected with HPV. Many studies have investigated the carcinogenic action of various animal papillomaviruses in both their natural and heterologous hosts. Studies in monkeys, cattle, rabbit, and sheep have shown that animal papillomaviruses cause cancer in their natural host. Transgenic mice studies have demonstrated that HPV proteins play a role in the development of dysplasia and progression to tumors. Forty-one percent of female transgenic mice containing HPV-18 LCR, part of E1, E6, and E7 genes developed cervical neoplasms at 1 to 2 years. Transgenic mice containing the HPV-16 early region developed stomach cancer. In another study, expression of the HPV-16 early region was targeted to squamous epithelial. These mice developed squamous epithelial neoplasia at multiple epithelial sites including the face, snout, eyelids, and anus; chronic treatment with estrogen induced cervical and vulvar carcinoma in these mice.

Genotoxicity, mechanistic, and other relevant data

Genotoxicity

High-risk HPV infection is associated with genetic instability; chromosomal aberrations, including tetrasomy, abnormal centrosome numbers, and chromosomal imbalances at chromosomal regions; and aneuploidy.

Mechanistic data

HPV can integrate into the host DNA, immortalize, and transform cells. Although the viral proteins E2 and E5, as well as the LCR, play a role in HPV-induced transformation,

the primary immortalization and transformation proteins are E6 and E7. Studies with transgenic mice further support the notion that E6 and E7 proteins are important in HPV-associated neoplasia. The E6 protein interacts with p53 by increasing the degradation of p53, thereby interfering with apoptosis. The E7 protein disrupts transcription factor complexes with pRb and related cell cycle control proteins. It also induces their degradation, altering transcriptional controls and cell cycle progression. Both the E6 and E7 proteins alter growth regulatory pathways by interfering with growth receptors or growth factors; cytokine expression was altered in HPV-16 positive cells. Expression of E7 causes mitotic abnormalities by inducing aberrant centrosome duplication. Most mechanistic studies have been conducted with HPV-16 and HPV-18, although some studies have utilized other viruses. The available mechanistic data on the individual viruses are presented in the table below.

Summary of the mechanistic evidence for HPV viruses

HPV type	Cell culture	Biochemical
HPV-16	Recombinant DNA immortalizes human foreskin keratinocytes and other cells, and E7 protein promotes anchorage-independent growth *in vitro*.	E6 oncoprotein promotes degradation of p53. E7 protein inactivates cell cycle regulatory proteins (pRb, p21, p27, p107, and p130).
HPV-18	Recombinant DNA immortalizes human foreskin keratinocytes and other cells.	E6 oncoprotein promotes degradation of p53. E7 protein inactivates cell cycle regulatory proteins.
Other (not 16 & 18) HPVs	Recombinant DNA (HPV-31 and 33) immortalizes human foreskin and cervical keratinocytes, E6 or E6/E7 genes (HPV-33) transform NIH3T3 cells, and E7 protein (HPV-32 and 77) promotes anchorage-independent growth *in vitro*.	E7 protein (HPV-32, 54, and 77) binds to cell cycle regulatory proteins (pRb, p107, and p130).
High-risk HPVs as a class (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)	na	na

Abbreviations Used in the HPV Background Document

ACIS	Adenocarcinoma *in situ*
ADC	Adenocarcinoma
ADS	Adenosquamous
AIDS	Acquired Immunodeficiency Syndrome
AF	Attributable fraction
ALIVE	AIDS Link to Intravenous Drug Experience
AMP	Adenosine monophosphate
ASCUS	Atypical squamous cells of undetermined significance
B7, B63	Class I HLA alleles
BPV	Bovine papillomavirus
BWSCC	Basaloid or warty squamous cell carcinomas
CFS	Common fragile sites
CgPV	Colobus monkey papillomavirus
CIN	Cervical intraepithelial neoplasia
COPV	Canine oral papillomavirus
CRPV	Cottontail rabbit papillomavirus
DKG	Diathermo-electrocoagulation of the ectopic epithelium and transformation zone
DLG	*Drosophila* tumor suppressor, discs large

DMBA	7,12-Dimethylbenz[*a*]anthracene, used as a tumor initiator
DMPA	Depot medroxyprogesterone acetate
E1-E8	Early human papillomavirus genes 1 through 8
E6AP	E6-associate protein
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EV	Epidermodysplasia verruciformis
FDA	U. S. Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
H^+-ATPase	Proton (hydrogen) pump
HBcAg	Hepatitis B virus core antigen
HBsAg	Hepatitis B virus surface antigen
HBsAb	Antibody against hepatitis B surface antigen
HC II	Hybrid capture assay, second generation
HIV	Human immunodeficiency virus
HK1	Human keratin-1 gene
HLA	Human leukocyte antigens
HOK	HPV-immortalized human oral keratinocytes
HPV	Human papillomavirus

HR	High risk
HSIL	High-grade squamous intraepithelial lesion
HSR	Homogeneously staining region
HSV-2	Herpes simplex virus type 2
HTERT	Protein subunit of the human telomerase enzyme
IARC	International Agency for Research on Cancer
ICC	Invasive cervical cancer
IR	Intermediate risk
IRF	Interferon regulatory factor
ISH	*In situ* hybridization
IUD	Intrauterine device
K14	Human keratin-14
KSCC	Keratinizing SCC
L1-L2	Late HPV genes 1 and 2
LCR	Long control region
Leu	Leucine
LOH	Loss of heterozygosity
LR	Low risk
LSIL	Low-grade squamous intraepithelial lesion
M2-PK	M2 pyruvate kinase

mCTLp	Memory cytotoxic T lymphocyte precursors
MHC	Major histocompatability complex
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
n	sample size
NCR	Noncoding region
NER	Nucleotide excision repair
NFI	Nuclear factor I
NHOK	Normal human oral keratinocytes
NIEHS	National Institute of Environmental Health Sciences
NOS	Not otherwise specified
OC	Oral contraceptive
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PAH	Polycyclic aromatic hydrocarbon
PAP	Peroxidase-antiperoxidase
Pap	Papanicolaou smear test
PCR-EIA	Polymerase chain reaction- enzyme immunoassay
pRb	Retinoblastoma tumor suppressor protein
Rb	Retinoblastoma

RhPV	Rhesus monkey papillomavirus
ROPV	Rabbit oral papillomavirus
RPV	Reindeer papillomavirus
RR	Relative risk
s.c.	Subcutaneous
SCC	Squamous cell carcinoma
SEER	Surveillance, Epidemiology, and End Results
SIL	Squamous intraepithelial lesion
SSCP	Single-strand conformation polymorphism
STD	Sexually transmitted disease
TGF	Transforming growth factor
TPA	Tetradecanoyl phorbol acetate
TSP	Thrombospondin
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling
URR	Upstream regulatory region
UVB	Ultraviolet B radiation
Val	Valine
VEGF	Vascular endothelial growth factor
VIN	Vulvar intraepithelial lesions
VLP	Viral-like proteins

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1 Introduction

Human papillomaviruses (HPVs) of the genital-mucosal associated types have been nominated for possible listing in the Report on Carcinogens by the National Institute of Environmental Health Sciences (NIEHS), based on an International Agency for Research on Cancer (IARC) finding of sufficient evidence of carcinogenicity in humans for HPV-16 and HPV-18 (Group 1) and limited evidence of carcinogenicity in humans for HPVs 31 and 33 (Group 2A). IARC also classified some HPV types other than 16, 18, 31, and 33 as possibly carcinogenic to humans (Group 2B) and noted that there was evidence suggesting lack of carcinogenicity to the cervix in humans of HPV types 6 and 11.

Human papillomaviruses (HPV) are DNA viruses that belong to the papillomaviridae family, which includes more than 100 types specific to humans and additional virus types that infect only specific animal species. HPVs infect the squamous epithelium of the skin and mucosal membranes. This document is restricted to the review of the genital-mucosal-associated types, which include more than 35 types that have been classified in the literature as either high risk or low risk. High-risk viruses are associated with cervical cancer, whereas low-risk viruses are associated with genital warts or low-grade cervical intraepithelial neoplasia. Most studies to date have considered HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 to be high-risk viruses (these are the ones identified by the Hybrid Capture II, second generation [HC II] assay) although some studies also include other HPVs, most notably HPV-66. Classification of viruses is also based on phylogenetic and mechanistic considerations. Most high-risk viruses share homology with HPV-16 and HPV-18. Mechanistic studies have shown that high-risk but not low-risk viruses immortalize human keratinocytes, interact with pRb and p53, and induce chromosomal aberrations. However, most mechanistic studies have evaluated only a limited number of viruses; the majority of the studies focused on HPV-16 and HPV-18, and only a few centered on HPV-31 and HPV-33. Some of the studies reported in this document evaluated individual HPVs, whereas other studies evaluated HPV (usually high-risk) as a class or as sub-groups of HPV viruses.

1.1 Papillomavirus taxonomy

The papillomaviridae family consists of DNA viruses that infect the epithelia of the skin and mucous membranes and induce a variety of benign and malignant tumors in humans and other species. Some members of this family are considered to be tumor viruses because of their close association with human and animal tumors and their ability to immortalize normal cells. Papillomaviruses have narrow host specificity and tissue specificity. The mechanism of species specificity has not been determined but is probably due to host regulatory proteins rather than to virus attachment and penetration. HPVs do not infect any animal hosts. Animal papillomaviruses, which are species specific, affect many species of domesticated or wild mammals as well as some species of birds.

1.1.1 General classification of papillomaviruses

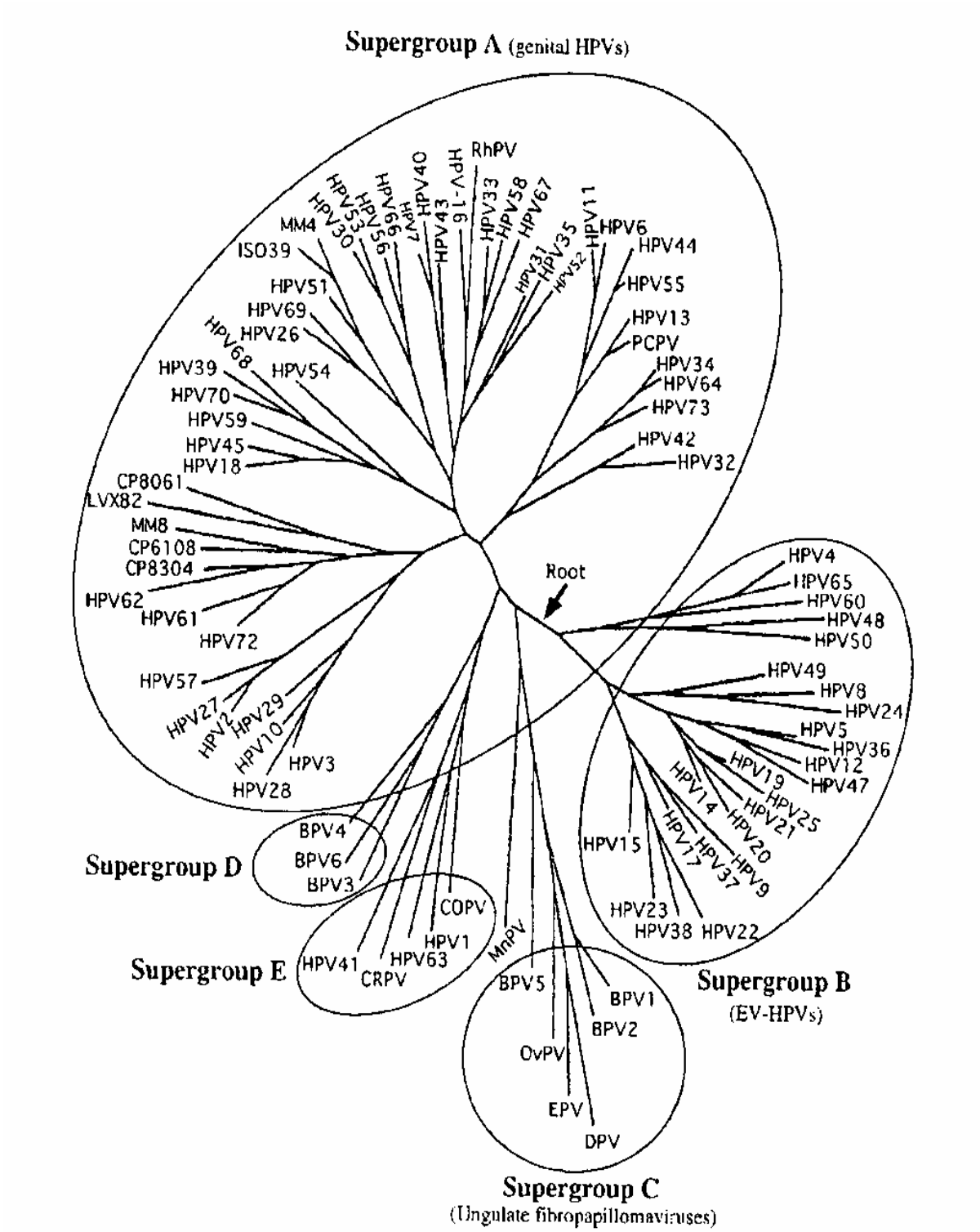
Classification of virus types is based on species of origin and homology between viral genomes. HPVs have been studied the most extensively and are defined by genomic

analyses, and thus, HPV types are genotypes. In contrast, animal viruses have not been studied as extensively, and only a few types are usually known for each species. Figure 1-1 depicts a phylogenetic tree in which animal and human viruses are grouped into five major Supergroups (A to E); the classification of viruses is based on a nucleotide sequence comparison of a segment coding for one of the capsid proteins (L1). Animal papillomaviruses are found in all five supergroups, whereas the 92 HPVs are only found in three supergroups. Thus HPVs are more closely related to animal papillomaviruses in their supergroup than to HPVs in a different supergroup (Howley and Lowy 2001).

1.1.2 Classification of HPVs

More than 100 HPVs types have been characterized to date, and there are probably new types yet to be identified. A new HPV type is defined as having < 90% sequence homology with other HPV types in the open reading frames (ORF) of certain viral proteins (E6, E7, and L1). HPV can be subdivided into three categories: cutaneous types (e.g., HPV-1, 2, 4), which cause nongenital warts; epidermodysplasia verruciformis (EV) types (e.g., 5, 8, 20, 22), which cause nongenital skin lesions in individuals with EV (a condition characterized by high susceptibility to HPV infection) or in immunosuppressed individuals; and genital-mucosal types (e.g., 6, 11, 16, 18), which are the focus of this document. The reasons for the specificity of these viruses for certain body sites are not known but that specificity does not appear to be mediated by cell-specific receptors (see Section 1.2.3.1). However, some researchers have speculated that intracellular regional-specific regulatory factors play a role (Lowy and Howley 2001, Howley and Lowy 2001).

The genital-mucosal types, which include over 40 types, infect the genital skin, and genital and nongenital mucosa. These viruses infect the anogenital tract including the vulva, vagina, cervix, anal canal, and penis, and also can infect the aerodigestive tract and oral cavity. The genital-mucosal HPVs cause a spectrum of clinical manifestations ranging from asymptomatic infections to genital warts to persistent infections that may lead to malignancy. HPV types that have been associated with cervical cancer are characterized as “high-risk” types (e.g. HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). As mentioned above and depicted in Figure 1-1, HPV types are found in three of the supergroups; genital HPVs, the focus of this document, are all found in Supergroup A (genital HPVs). Most of the high-risk types are found in two clusters, one containing HPV-16 and one containing HPV-18. “Low-risk” types (e.g., HPV-6, 11, 53, 61, 70, and 71) are primarily associated with low-grade squamous intraepithelial lesions, recurrent respiratory papillomatosis, and genital warts (mainly HPV-6 and 11) (Lowy and Howley 2001, Howley and Lowy 2001).



Source: Chan *et al.* 1995 with permission.

Figure 1-1. Phylogenetic tree for 92 human and animal papillomaviruses

1.2 Structure and molecular biology

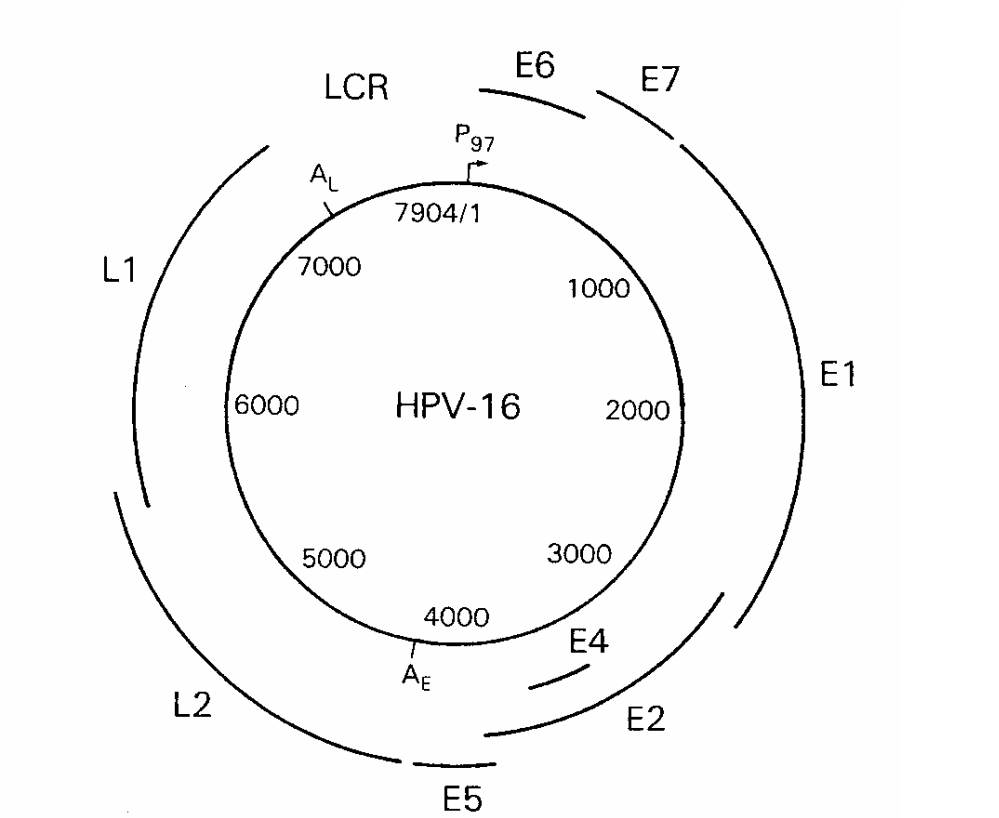
1.2.1 Virion structure

Papillomaviruses are small, nonenveloped icosahedral particles ~52 to 55 nm in diameter. The virion has 72 capsomers (60 hexameric + 12 pentameric) arranged on a T = 7 lattice. The viral capsid is made up of 2 proteins, 1 major (encoded by the L1 gene) and 1 minor (L2). The major capsid protein, L1, appears to be sufficient for particle formation. Capsomers consist of a trunk with distal and proximal thickening. They associate at their base and project radially. They exhibit a 5-fold symmetry and are therefore composed of five identical molecules, most likely molecules of the L1 protein, which makes up about 90% of the total protein. They are resistant to organic solvents and heating at 56ºC. Virus particles have a buoyant density of 1.34 g/mL in cesium chloride and a sedimentation coefficient of 300 (Howley and Lowy 2001).

1.2.2 Genome structure and organization

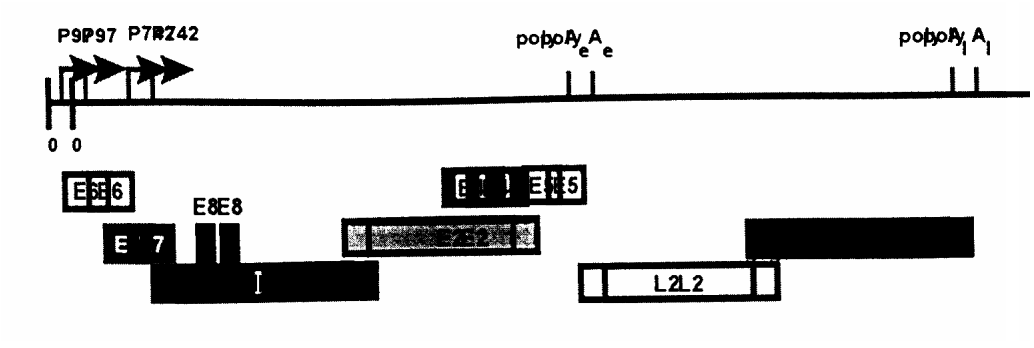
The HPV viral genome consists of 8,000 base pairs of covalently closed, double-stranded DNA and has ten functional units, referred to as ORFs, two late genes and eight early genes of which six early genes are illustrated in Figure 1-2. A seventh early gene ORF (E8) is contained within E1 as illustrated in Figure 1-3. The eighth early gene ORF (E3) is largely coincident with E4 and has no known function; this ORF is not included in either figure. The small size of about 8,000 base pairs translates into relatively few viral-specific functions. Each function involves a defined part of the total genomic sequence and is responsible for the production of a specific protein. The functional units are read as a specific unit by the RNA polymerase. These ORFs are encoded by only one of the two DNA strands (the sense strand) and constitute 69% of this DNA sequence. The region not associated with any ORF is referred to as the long control region (LCR) and contains the origin of replication and regulatory elements (Howley and Lowy 2001).

Table 1-1 summarizes the functions of the HPV genes. Each ORF has been assigned a name according to its size and position within the circular genome and is classified as belonging in the early (E) or late (L) region. The early region of the genome (E1-E8) encodes regulatory proteins, including those involved in transcription and replication that are expressed both in nonproductively infected cells and in transformed cells. The E1 ORF encodes a critical protein for genomic replication; E2 encodes a DNA-binding, transcriptional regulatory protein and also is required for genomic replication. The E3 protein has no known function, and the function of the E4 protein is not well understood, but it has been shown to disrupt the cytokeratin structure. The E5 protein is membrane associated and has been shown to activate growth receptors such as epidermal growth factor receptor and platelet-derived growth factor receptor. The E6 and E7 proteins affect cellular proliferation and can immortalize and transform primary cells. The eighth ORF, E8, like E3, has been described as having no known function; however, recent publications suggest that E8 may act together with E2 to regulate HPV DNA replication (Stubenrauch *et al.* 2000, 2001). The late ORF (L1 and L2) encode proteins that make up the capsid structure; they are produced late in the infectious cycle of the virus (Howley and Lowy 2001).



Source: Shah and Howley 1996 with permission.

Figure 1-2. The genomic map of HPV-16



Source: Stubenrauch and Laimins 1999 with permission.

Figure 1-3. Transcription map of HPV-31

Table 1-1. Functions of HPV genes

Gene designation	Functions
E1	Initiation of viral DNA replication
E2	Regulation of viral transcription with an auxillary role in DNA replication
E3	No known function
E4	Disrupts cytokeratins
E5	Transformation (animals only)
E6	Transformation, targets degradation of p53 tumor supressor protein
E7	Transformation, binds to the retinoblastoma protein
E8	No known function
L1	Major capsid protein
L2	Minor capsid protein

Source: Howley and Lowy 2001.

1.2.3 Replication

Papillomaviruses specifically target squamous epithelial cells, and productive infection of cells is linked to the differentiation state of epithelial cells. Viral replication can be divided into early stages and late stages. Early stages (including attachment, entry and uncoating, early gene expression and protein production, and DNA replication) occur in basal cells because these are the only dividing cells in the squamous epithelium. Late stages include events leading to production of viral particles (late gene expression, production of capsid proteins, vegetative viral DNA replication, virus assembly, and release) occur in the terminally differentiating squamous epithelial cells (Howley and Lowy 2001).

1.2.3.1 Early stages

The first step of infection is the binding of HPV to a receptor on the basal keratinocytes. Receptors do not appear to be cell specific, and HPVs are capable of binding to other types of cells. Receptors have not been identified; one candidate receptor is the $\alpha 6\beta 4$ integrin, but HPVs also can bind to heparin and cell-surface glycosaminoglycans on human keratinocytes. After binding to the cell, the virions are transported to the nucleus, and the HPV DNA is uncoated (Howley and Lowy 2001).

Figure 1-3 shows a simplified (linearized version of the genomic map that includes the location of the promoters) transcription map of HPV-31, as representative of genital HPVs. Transcription is complex due to the presence of multiple promoters, alternate and multiple splice patterns, and differential production of mRNA in different cells. Transcription is regulated by the differentiation state of the squamous epithelial cell. The

early promoter, P97, initiates transcription of E6, E7, and other early genes, which are expressed in nonterminally differentiated cells in the lower portion of the epithelium. The late promoter, P742, directs expression of late gene products, including E4, L1, and L2, which are expressed in cells that have undergone differentiation. High-risk and low-risk viruses differ in the manner in which E6 and E7 are expressed. In the high-risk types (HPV-16, 18, and 31), expression of E6 and E7 is controlled by a single promoter, while the expression of these genes is regulated by additional early promoters in low-risk types (HPV-6 and 11). For high-risk viruses, E6 and E7 are transcribed to either a single E6-E7 mRNA or with splices in the E6 gene (Howley and Lowy 2001).

Enhancer motifs in the LCR respond both to cellular and viral transcription factors. The LCR also contains constitutively expressed elements that are cell-type specific. The constitutive enhancer elements are believed to be needed for initial transcription upon viral infection and for maintaining viral latency. This region also includes binding sites for E2 regulatory proteins and the origin of DNA replication (Howley and Lowy 2001).

1.2.3.2 Viral replication

Three modes of papillomavirus DNA replication have been described. The first phase of replication results in the amplification of the viral genome to 50 to 100 copies and occurs during initial infection of the basal keratinocyte. The second phase, genome maintenance, which is needed to ensure a persistent and latent infection of the stem cells, also occurs in the basal cells. The genome is replicated once per cell cycle during S phase. The third type of replication, vegetative DNA replication, is needed to generate genomes that will be packaged into virions; this phase occurs during the late stage of replication in terminally differentiated cells. Virus replication involves E1 and E2 proteins (Howley and Lowy 2001).

1.2.3.3 Late stages

Late stages include late gene expression, production of capsid proteins, vegetative viral DNA replication, virus assembly, and release. As mentioned above, late gene expression is initiated by the late gene promoter and includes the expression of E4, L1, and L2. Although E4 is located in the early region of the viral genome, it is considered a late protein because it is expressed from the late promoter and plays a role in productive infection. The E4 protein may contribute to vegetative DNA replication, or it may facilitate viral release through its role in the disruption of cytokeratin network. Little is known about virus assembly or release. The virus is not believed to be cytolytic (Howley and Lowy 2001).

1.3 Summary

HPVs are DNA viruses belonging to the papillomaviridae family, which includes more than 100 types specific to humans and additional types that infect only specific animal species. HPVs infect the squamous epithelium of the skin and mucosal membranes; the genital-mucosal-associated types include viruses classified as high-risk for their prevalence in invasive cervical cancer. The high-risk HPVs include HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, although some studies include others as well, most notably HPV-66. Other genital-mucosal types are considered to have low risk and are associated with genital warts or low-grade cervical cancer.

HPVs are small viruses consisting of approximately 8,000 base pairs of covalently closed, double-stranded DNA. The viral genome is divided into a series of ORFs that encode the relatively few viral-specific proteins and an LCR that contains the origin of replication and regulatory elements. The early genes are designated E1 through E8 and are associated with regulation of transcription (E2) and cellular proliferation (E6 and E7). The late ORFs (L1 and L2) encode proteins that make up the viral capsid structure and are produced late in the infectious cycle of the virus.

Papillomaviruses specifically target squamous epithelial cells, and productive infection of cells is linked to the differentiation stage of epithelial cells. The early stages of infection, which include the events from cell attachment and entry to viral DNA replication, can occur only in the dividing cells of the basal squamous epithelium. The late stages include events leading to production of viral particles and occur in terminally differentiated squamous epithelial cells.

2 Human Exposure

HPV infection is one of the most common sexually transmitted infections worldwide. This section discusses issues related to human exposure to HPV, including detection methods; issues related to the epidemiology of HPV infection, including prevalence, risk factors, transmission and acquisition and clearance of HPV infections; and prevention or treatment of HPV infections.

2.1 Detection of HPV infection

The HPV virus cannot be propagated or isolated from tissue-culture cells, probably because it requires differentiating cells to replicate. Thus, HPV infection must be inferred from visual or clinical observation of clinical lesions (warts or flat cervical lesions), microscopic detection of abnormal cytology, molecular diagnosis (HPV DNA), or serological responses (Trofatter 1997).

2.1.1 Visual or clinical inspection

HPV infection can be manifested as clinically apparent disease, such as distinctive anogenital warts (condylomata acuminata) or as flat condylomata; however, most infections do not produce visible lesions. Condylomata acuminata are genital lesions with a fleshy red appearance and a raised surface that usually extends in fingerlike projections and are visible to the naked eye (Trofatter 1997). Cellular changes in these warts are limited to the superficial layers of the epithelium and include koilocytic atypia (see below), multinucleation, and individual cell keratinization (Arends *et al.* 1998).

Flat condylomata, which are flat nonpapillary lesions, are more difficult to detect and may only be apparent by colposcopic examination after acetic acid treatment, in which the infection appears as a white, flat, shiny lesion. Colposcopy is used to localize the lesions. However, these lesions are not unique to HPV infections; thus the specificity of this test is low (Arends *et al.* 1998, Trofatter 1997). Clinical diagnosis cannot distinguish HPV types.

2.1.2 Light microscopy (cytologic and histologic methods)

The Papanicolaou (Pap) smear, which involves microscopic observation of stained exfoliated genital cells, detects koilocytosis and cervical intraepithelial neoplasia (CIN). Koilocytic cells have a characteristic appearance marked by a dark abnormal nucleus encircled by a clear cytoplasmic ring and collapse of the cytoskeleton; they are considered part of the lowest grade of CIN (CIN I). The appearance of these cells is considered diagnostic of an HPV infection; however, not all HPV-infected individuals have detectable koilocytosis. Thus, the sensitivity of koilocytosis for detecting HPV infection is low (Trofatter 1997).

Squamous epithelial cell abnormalities are considered suggestive of HPV infection, CIN II and III, or both. The Pap smear also is used to screen for cervical cancer because it can identify CIN, which may progress to cervical cancer (Kahn 2001). Although the rates of

cervical cancer have significantly declined since the implementation of the Pap smear, the accuracy of a single test is limited; the Pap smear has been reported to miss 20% to 30% of high-grade squamous intraepithelial lesions (CIN) or cancer and to have a false positive rate of 7% to 27% (Wick 2000). The use of additional tests in triage screening programs, such as HPV-DNA testing, may increase the sensitivity for detecting cervical abnormalities (see Section 2.1.3.1). Individuals with a positive Pap smear are usually referred for colposcopic exam (Kahn 2001).

Although HPV infections have been inferred through visual exam, colposcopy, histology, and cytology, these techniques were not able to detect the virus itself, but merely the clinical manifestation of HPV infection and, thus, they also fail to discriminate among various HPV types.

2.1.3 Molecular diagnosis (detection of HPV DNA)

The most sensitive and specific method to detect HPV infection is through DNA testing. Moreover, this method also provides for the detection of a broad spectrum of DNA genotypes (Trofatter 1997). Detection of HPV DNA signifies a present exposure or persistent infection resulting from a past exposure (Dillner 2000). The most sensitive HPV DNA methods include PCR-based methodology and the Hybrid Capture (HC) assays (Trofatter 1997). HPV DNA detection also is dependent on the quality of the specimen, with biopsies sometimes resulting in higher rates of HPV positivity than exfoliated cells (Bosch *et al.* 1997).

2.1.3.1 Signal amplification methods

Hybrid Capture assays are the most commonly used signal amplification methods, and these assays are the only HPV DNA tests currently approved by the FDA (Peyton *et al.* 1998, Digene Corporation 2001). An important advantage of these assays is that they only require one step to identify the virus as either high or low risk. There are two types: HC, which is no longer used, and the HC II.

2.1.3.1.1 Hybrid Capture assay

The HC assay is based on the formation of RNA-DNA hybrids between HPV DNA and unlabeled HPV RNA probes. The hybrids are captured and immobilized by antihybrid antibodies and are reacted with a monoclonal antibody reagent that is conjugated to alkaline phosphatase. The complexes are detected with a chemiluminescent substrate reaction, using a tube luminometer (Peyton *et al.* 1998). The HC assay detects high- and low-risk HPV types using two RNA probe pools, or cocktails: probe A for five low-risk HPV types (6, 11, 42, 43, and 44), and probe B cocktail for nine high-risk HPVs (16, 18, 31, 33, 35, 45, 51, 52, and 56). One disadvantage of the HC assay is cross-hybridization of both the low- and high-risk probe cocktails with HPV types not represented in the cocktails but genetically similar to high-risk HPV types (Vernon *et al.* 2000). This method has greater sensitivity (using PCR as the gold standard for HPV status) for detecting HPV infections in women with abnormal cytology (81%) than in women with normal cytology (47%) (Cope *et al.* 1997). The HC assay is no longer widely used due to the introduction of the HC II assay.

2.1.3.1.2 Hybrid Capture II assay

The HC II assay follows the same basic technique as that used in the HC assay, except that in the newer version a microplate luminometer is used to read the light output and display the assay results as relative light units. A comparison of the relative light units to a standard positive reference is used to determine whether a specimen is positive or negative for HPV. Probes for HPV types 39, 58, 59, and 68 have been added to probe pool B for high-risk HPV types (16, 18, 31, 33, 35, 45, 51, 52 and 56), raising the total number of viruses to 13. A 1.0 pg/mL cutoff can be used in this test, which is approximately a 10-fold increase in sensitivity over the HC assay. A study comparing the HC II test to PCR using the MY09/MY11 consensus primers reported an approximately 90% agreement when the data were restricted to HPV types found in the A and B probes (using the 1.0 pg/mL cutoff) (Peyton *et al.* 1998).

The HC II assay has been proposed as a screening test for cancer prevention, either in addition to the Pap smear (cytology) or in a triage program using the HPV DNA test as the primary screen and the Pap smear to monitor HPV DNA-positive subjects. Cuzick *et al.* (2001) reviewed several studies that compared HPV DNA testing using the HC II assay with cytology testing for their ability to predict high-grade cervical lesions (CIN II and III). Higher sensitivities were reported for HPV DNA testing (81% to 100%) than for cytology (Pap smear) (44% to 86%); however, the opposite pattern was observed for specificity, which was slightly higher for cytology testing (Cuzick 2001).

2.1.3.2 Target amplification methods

Polymerase chain reaction (PCR)-based methods, which amplify a targeted segment of DNA sequence, are commonly used in HPV cancer epidemiology studies. The amplified DNA can be detected by electrophoresis and staining of the agarose gels (ethidium bromide), or through detection with a labeled probe (Trofatter 1997). The major advantages of PCR assays are their high sensitivity and their specificity. The sensitivity of the assay is dependent on the primer and detection methods for DNA; sensitivities were in the femtogram to picogram range for ethidium bromide detection and 10 times more sensitive for probe-based detection after Southern hybridization of the amplified product (Harnish *et al.* 1999). PCR primers can be type specific or general (consensus); that is, they can amplify many HPV types (Trofatter 1997).

2.1.3.2.1 Consensus primers – amplification of HPV DNA

Most PCR assays for HPV-DNA detection use consensus primers to amplify a broad spectrum of HPV types, followed by a second step, such as hybridization with HPV-specific probes, to type the DNA (Jacobs *et al.* 1995). Numerous consensus primers have been developed, with the majority designed to amplify highly conserved regions of either the L1 or E1 ORFs (Wick 2000). Most of the cancer epidemiological studies reviewed in Section 3 use the MY09/MY11 or GP5+/GP6+ consensus primers, which are located in a highly conserved region of the L1 ORF. The MY09/MY11 primers, which amplify a 450-base segment, were originally designed from a consensus sequence from the five HPV genotypes whose sequences were available at that time but which are capable of amplifying more than 30 genital types (Manos *et al.* 1989, Gravitt *et al.* 2000, Laconi *et al.* 2001). However, the sensitivity for detecting HPV DNA varies according to HPV type

as a result of the number and position of mismatches in the degenerate primers. The primers have been modified (by addition of an HPV-51 specific oligonucleotide) to improve the sensitivity for amplifying one of the cancer-associated HPVs (HPV-51) (Gravitt *et al.* 2000). Further improvements of these consensus primers are likely to be made in the future. For example, Gravitt *et al.* (2000) recently redesigned the primers to eliminate the degenerate base design. Sequence heterogeneity was achieved by using multiple primers (with sequences from the same region as the MY09/MY11 primers), which were combined into upstream and downstream pools (PGMY09/PGMY11). The PGMY09/PGMY11 system appeared to be more sensitive than the MY09/MY11 system in an initial study of 262 specimens; moreover, the system could detect a greater number of multiple infections (Gravitt *et al.* 2000).

The GP5/GP6 primers were designed from a highly conserved region in L1 that was homologous in nine HPV types for which sequence data were available. These primers generate smaller amplicons (~150 base pairs) than the MY09/MY11 primers; thus, they are more efficient with archival (e.g., paraffin-embedded) tissue (Snijders *et al.* 1990). de Roda Husman *et al.* (1995) improved the efficiency of amplification by elongating the primers at their 3' ends (three nucleotides to the GP5 primer and five nucleotides to the GP6 primer). These primers, GP5+/GP6+, had 10-fold to 100-fold higher sensitivity than the GP5/GP6 primers and had an increased signal-to-background ratio. The GP5+/GP6+ primers can amplify most of the HPV types found in genital tissue (Jacobs *et al.* 1997).

Other consensus primers have been designed from the L1 region and from conserved regions in the E1 (for example, the CPI/II primers) and the E6 and E7 ORFs (for example the LCR-E7). The E6 and E7 regions are important domains for binding the tumor-suppressor proteins, p53 and pRb (see Section 6) (Walboomers *et al.* 1999, Sasagawa *et al.* 2000, Harnish *et al.* 1999). A comparison of consensus primers has shown that the primers vary in sensitivity and specificity for detecting HPV from cervical samples; thus, the use of more than one set of consensus primers is recommended for screening samples for HPVs (Harnish *et al.* 1999).

2.1.3.2.2 Typing of DNA

After HPV DNA is amplified from a sample, the HPV is genotyped by one of many methods, including PCR with type-specific primers, hybridization with HPV-specific probes (dot blot or Southern blot), restriction length polymorphism analysis, sequencing, and enzyme immunoassays (Jacobs *et al.* 1995). Jacobs *et al.* (1995) designed a typing assay to distinguish high-risk from low-risk types for situations in which genotyping of individual viruses was not needed. HPV DNA was amplified using the GP5+/GP6+ consensus primers (discussed above) and typed as high or low risk by hybridizing the PCR products to two cocktails of type-specific oligonucleotides; the sensitivities of the oligonucleotide probes varied from picogram level to femtogram level depending on HPV type.

Other investigators have attempted to decrease the labor and time requirements involved in HPV genotyping. Laconi *et al.* (2001) developed a genotyping assay that incorporates the use of a labeled consensus primer (GP5+/GP6+ primers) in the initial PCR amplification step, followed by reverse hybridization to HPV-specific probes, capture on

plastic microwells, and detection by immunoenzymatic assay. Gravitt *et al.* (1998) developed a reverse-blot method, in which the biotin-labeled PCR products (amplified by the MY09/MY11 primers) were hybridized to a filter strip containing an array of 27 immobilized oligonucleotide probes, thus allowing the typing of multiple HPVs in a single hybridization reaction.

2.1.3.3 Nonamplification methods

Southern blot analysis of HPV, which is based on the hybridization of HPV-specific probes to specimen DNA that has been digested with restriction enzymes and separated by molecular weight, is labor intensive and requires large quantities of DNA (5 to 10 μg); thus, it is seldom used in studies today (Walboomers *et al.* 1994, IARC 1995). Southern blot analysis is sometimes used in epidemiological studies to genotype HPV DNA after amplification by consensus primers and as a crude assessment of viral load. Because larger quantities of DNA are needed for Southern blot analysis than for PCR assays, high viral load is assessed as being positive only by both Southern and PCR analyses, whereas low viral load is assessed as positive by PCR methodology alone.

Dot blot analysis consists of fixing DNA onto a membrane rather than using electrophoresis. Smaller quantities of DNA (0.3 to 1.0 μg) are required than for Southern blot analysis; however, as with Southern blot analysis, it is very difficult to differentiate between related HPV types. The ViraPap assay, which has been supplanted by the more sensitive HC II assay, was a type of dot blot analysis that contained RNA probes for a limited number of HPV types: 6, 11, 16, 18, 31, 33, and 35 (Walboomers *et al.* 1994, IARC 1995).

2.1.4 Serological biomarkers

Most HPV antibodies to virus-like particles in human sera are neutralizing and are type specific with the exception of anti-HPV-6 and 11, which cross-react. The major isotypes of serum antibodies against capsid antigens are IgG1 and IgA, but the IgA response may be much lower than the IgG response. Type-specific secretory IgA antibodies and local IgG antibodies have been found in cervical mucus and/or secretions (Dillner 2000).

Serological assays to measure antibodies (IgG) against capsid antigens (most often tested as virus-like particles) were established in 1994. These assays usually measured antibody titers using the enzyme-linked immunosorbent assay (ELISA) (Dillner 2000). Seroepidemiological studies discussed in Section 3 have assayed antibodies to HPV types 6, 16, 18, and 33, although assays have been developed for other HPV types, as well. Assays also have been developed using antibodies against E6 and E7, but these have not been used as extensively. E6 and E7 antibodies are associated with invasive cancer and tumor burden and do not correlate with capsid antibodies (Sun *et al.* 1999).

Detection of HPV antibodies signifies present or past exposure. Seroprevalence is strongly related to lifetime number of sexual partners, but less so to the number of recent partners (Dillner 1999). De Gruijl *et al.* (1997) reported that significantly higher proportions of patients with persistent HPV infections (69%) were positive for the

presence of HPV-16 antibodies than were patients who cleared HPV-16 infections. In some individuals, seroconversion may be delayed many months after DNA detection (Dillner 2000).

Several validation studies have estimated the sensitivity of HPV serology tests to be approximately 50% using detection of HPV DNA as a standard (Dillner 2000). Individuals with transient infections are less likely to seroconvert (HPV-16) than those with persistent infections; however, some women with persistent HPV DNA failed to seroconvert, which may be explained by differences in the host genetic background or immune status (Carter *et al.* 2000). Some type-specific differences in antibody responses were observed in a longitudinal follow-up study of 588 college women: seroconversion for HPV-16 usually occurred 6 to 12 months after HPV DNA detection, whereas seroconversion for HPV-6 usually coincided with HPV detection. Moreover, antibody responses to HPV-16 and 18 were more likely to persist than antibody responses to HPV-6 (Carter *et al.* 2000). Serological assays are not recommended for diagnosis in individual women due to low sensitivity, but they are useful in comparisons of groups in epidemiological (see Section 3) and ecological studies (Dillner 1999).

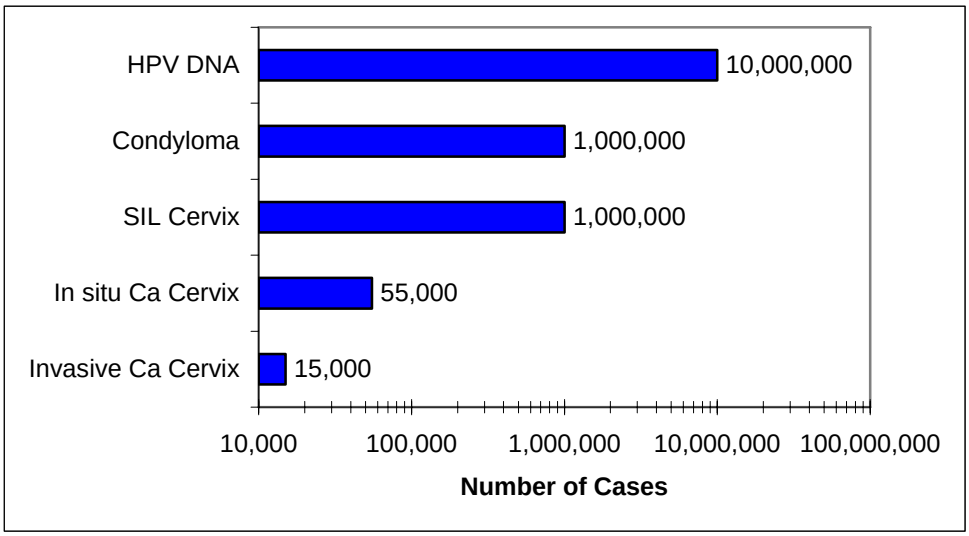
2.2 Prevalence

2.2.1 Prevalence in genital tract

Prevalence estimates depend on many factors, including the method of assessment of HPV infection and demographic and behavioral profiles of the subjects.

As discussed in Section 2.1, HPV infections can be asymptomatic (usually assessed by HPV DNA), may be manifested as anogenital warts (condyloma), or may be associated with CIN and cervical cancer. Crude prevalence estimates for HPV genital infections and HPV-associated diseases are depicted in Figure 2-1. The majority of HPV infections appear to be asymptomatic, with approximate 10 million cases reported in women in the United States per year compared to 1 million cases of anogenital warts and 1 million cases of cervical neoplasia. Koutsky (1997) reported an estimated prevalence of HPV infection as assessed by molecular evidence (HPV DNA) to be approximately 10% to 20% in men and women 15 to 49 years of age; lower prevalence estimates were observed for genital warts (1%) and for HPV-associated abnormal cytology (4%).

Prevalence estimates are highly dependent on the demographic and behavior profiles of the study population, with the highest estimates being observed in young, sexually active individuals (see Section 2.3 for a discussion of risk factors associated with HPV infection). Epidemiological studies in the United States have reported the prevalence of HPV DNA to be between 25% and 40% in sexually active young women, aged 15 to 25 years (Lowy and Howley 2001).



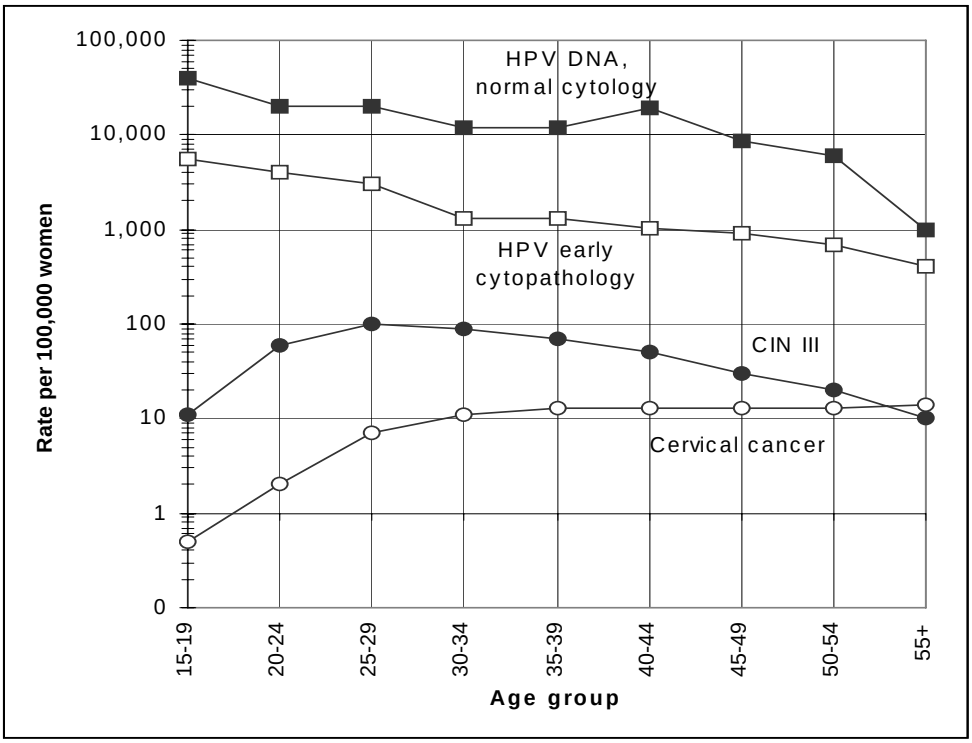
Source: Lowy and Howley 2001 with permission

Figure 2-1. Prevalence estimates of genital tract HPV infections and of HPV-associated diseases in women in the United States

Estimates of invasive and *in situ* carcinoma cases are from the American Cancer Society; other estimates are crude and derived from many sources.

Prevalence estimates of HPV infections also are age dependent. Most studies have shown that HPV DNA detection decreases after age 25; estimates are usually between 5% and 15% in most populations of mixed age groups. The decline in prevalence with age does not appear to be only the result of changing sexual behaviors. It also may be due to immune status; humoral immunity may play a role in the clearance and possibly resistance to HPV infections (Herrero and Muñoz 1999). Several studies have reported a second peak in prevalence of HPV DNA in women over 55 (Herrero *et al.* 2000, Lazcano-Ponce *et al.* 2001).

The relationship between age and HPV prevalence appears to be dependent on the type of HPV infection (i.e., normal cytology or clinical disease). Figure 2-2 shows age-specific rates of cervical HPV infection and associated disease in the United States (prevalence rates for HPV DNA with normal cytology and HPV early cytopathology and incidence rates for high-grade cervical disease [CIN III] and cervical cancer). Age-specific prevalence rates for both HPV DNA in women with normal cytology and HPV early cytopathology decrease with age: the incidence of CIN increases with age until age 25 to 29, then decreases; and the incidence rate of cervical cancer increases with age up to age 30 and then appears to plateau (Schiffman 1992). See Section 3.1 for a discussion of incidence rates of cervical cancer.



Source: Lowy and Howley 2001 with permission

Figure 2-2. Age-specific rates of cervical HPV infection and associated disease in the United States

Point prevalence of HPV DNA in cytologically normal women is based on data from 463 women screened between 1989 and 1990 at the Kaiser-Permanente clinic in Portland, Oregon. Point prevalence of HPV early cytopathologic effects is based on data from 19,571 women who submitted Pap smears at the Kaiser-Permanente screening program. The incidence of CIN III and cervical cancer are from the National Cancer Institute Surveillance, Epidemiology and End Result program cancer registries (1984 to 1988) for white women.

As discussed in Section 1, more than 40 HPV types can infect the genital tract; however, only a subset of these, designated as high-risk, have been associated with cervical cancer. The distribution of HPV type is dependent on the diagnosis. In normal cervical tissue, the distribution of high- and low-risk types is similar; however, with increasing severity of disease, the frequency of high-risk types increases dramatically (Lowy and Howley 2001). In a study of women 18 to 40 years of age, with no history of high-grade cervical disease, Peyton *et al.* (2001) reported the prevalence of HPV to be 39%, high-risk types to be 26.7%, low-risk types to be 14.7%, and uncharacterized types to be 13.0%. HPV-16 was the most common type found, occurring in 7.5% of the women. The distribution of HPV types also can vary slightly by geographic area (Koutsky 1997). While HPV-16 appears to be the most prevalent type worldwide, the distribution of some of the other viruses can vary; for example, HPV-45 is more prevalent in Western Africa, HPV-39 and 59 are more dominant in Central and South America, and HPV-52 and 58 are more prevalent in the Pacific Basin (Jastreboff and Cymet 2002).

Few studies have assessed HPV prevalence in men. Castellsagué *et al.* (1997) reported the prevalence of HPV DNA from husbands of women enrolled in four cervical case-control studies of CIN III and invasive cancer from Colombia and Spain. HPV DNA prevalence in the husbands of cases (from penile cell samples) was somewhat similar in both countries (18% in Spain and 26% in Colombia) and higher than that observed in controls. However, the prevalence of HPV in controls from Colombia (19%) was 5-fold higher than from Spain (3.5%) and was higher in cases from both countries than in controls and HPV DNA prevalence in Spain, which is consistent with the 8-fold higher incidence of cervical cancer in Colombia. High-risk HPV types were found at a higher prevalence than low-risk HPVs in the husbands of the cases from both countries. Franceschi *et al.* (2002) reported the results from an analysis that combined the data from five case-control studies of invasive cervical cancer and two studies of cervical carcinoma *in situ*, all conducted by IARC. HPV DNA (isolated from exfoliated cells from the penis) was found in 13% of the husbands of control women (n = 533), 18% of the husbands of women with invasive carcinoma (n = 445), and 21% of the husbands of women with carcinoma *in situ* (n = 165). HPV DNA prevalence (husbands of control women) varied greatly by geographical location, ranging from 3% in Spain to 39% in Brazil. The same HPV was seldom detected in both husband and wife.

2.2.2 HPV prevalence in non-anogenital tissue

HPV DNA has been detected in oral cells and other cells from tissue related to the head and neck regions. Case-control studies of head and neck-related cancers described in Section 3 (Appendix A, Table 5) have reported that prevalence (percent) of HPV DNA in controls ranges from 0% to 20%, albeit the estimates are not comparable across studies due to differences in tissue sites and HPV type. Moreover, most of these studies were based on small numbers and thus probably provide imprecise estimates. Terai *et al.* (1999) reviewed studies on the oral cavity of adults and reported HPV DNA rates between 12% and 60% depending on the patient population and detection method. HPV DNA also has been detected in head and neck-related cancers (Section 3).

2.2.3 HPV seroprevalence

HPV infections are usually transient and are characterized by a high rate of spontaneous clearance (70% clearance rate on a 12-month follow-up); thus, a point detection of HPV DNA in women without cancer usually represents a present exposure (Dillner 2000). Some ecological studies suggest that HPV seroprevalence rates correlate better with geographical cervical cancer rates than do HPV DNA prevalence estimates (Dillner 2000, Strickler *et al.* 1999). Seroprevalence also varies with the population. Most of the seroepidemiological studies discussed in Section 3 found seroprevalence (HPV-16, 18, or 33) to be in the range of 5% to 20% in the control population. Strickler *et al.* (1999) reported the age-adjusted HPV-16 seroprevalence rates in the United States to be 12% in female blood donors. The NHANES-III survey reported that 18% of women and 8% of men in the United States have HPV-16 antibody.

2.3 Risk factors associated with HPV infection

Sexual activity is the strongest predictor of genital HPV infection. As mentioned above, prevalence of HPV infection is highest for sexually active young adults between 15 and 25 years of age. Numerous studies in women have reported a positive relationship between lifetime number of sexual partners and HPV seropositivity (Silins *et al.* 2000, Sun *et al.* 1999) or detection of HPV DNA (Kjær *et al.* 1997, Lazcano-Ponce *et al.* 2001, Franco *et al.* 1995). Recent sexual activity, as assessed by the number of sex partners, frequency of sexual intercourse, and presence of genital warts on sex partners, is a strong predictor of HPV DNA (Franco *et al.* 1995, Ho *et al.* 1998a). Other risk factors include the sexual activity behaviors of the women's husband or sex partner. Thomas *et al.* (2001a) reported that the risk of invasive cervical cancer in monogamous women in Thailand increased with the number of lifetime visits their husbands made to prostitutes.

Risk factors in males are similar to those in women. Several studies have shown that the most important predictors for HPV DNA (penile) are lifetime number of sex partners, young age, and being uncircumcised (Svare *et al.* 2002, Castellsagué *et al.* 1997, 2002). Castellsagué *et al.* (1997) reported that penile prevalence for HPV was higher in husbands of nonmonogamous women than in husbands of monogamous women for each category of the number of sex partners of the husbands. Male circumcision was associated with reduced risk of penile infection and reduced risk of cervical cancer in female sex partners in a pooled analysis of seven case-control studies in five countries (Castellsagué *et al.* 2002).

Several studies have investigated whether risk factors for HPV infection differ for high- and low-risk HPV viruses; some (Franco *et al.* 1995, Richardson *et al.* 2000), but not all, (Lazcano-Ponce *et al.* 2001, Rousseau *et al.* 2000) reported that the correlation with sexual behavior is weaker for low-risk types, suggesting differences between correlates of transmission based on risk category (Franco *et al.* 1995, Richardson *et al.* 2000).

Current high-risk HPV infection may influence the risk for acquiring a new infection. Liaw *et al.* (2001) reported that a preexisting infection of HPV-16 might have increased the risk for a subsequent infection of some, but not all, HPV types; however, HPV-16 may be a marker for sexual exposure and may represent greater exposure to other HPV types. HPV-16 had no effect on the persistence of concomitant infections.

2.4 Transmission

Genital HPVs are primarily transmitted through sexual contact with infected cervical, vaginal, vulvar, penile, or anal epithelium (IARC 1995). This finding is supported by numerous epidemiological studies demonstrating that HPV infection is associated with behaviors related to sexual activity (Section 2.3).). The role of men as vectors of HPV infection has been demonstrated in studies showing that cervical cancer is increased in wives whose husbands have detectable HPV DNA in their penis, and who have a higher number of extramarital partners (Bosch *et al.* 1996). Penile lesions containing high-risk HPV DNA are frequent in male sexual partners of women with CIN (Bleeker *et al.* 2002). Moreover, HPV DNA was detected in 23% of semen of male partners of women with histologically detected genital HPV infections. In this study, HPV was detected

more frequently in men with penile or urethral lesions than in men without such lesions, suggesting that the mechanism of semen contamination by HPV may be exfoliation of infected cells from urethral lesions during semen ejaculation, or abrasion from penile lesions (Aynaud *et al.* 2002).

There are conflicting reports concerning whether HPV is transmitted at birth or perinatally. Studies have reported variable HPV detection rates in infants born to women with (1.5% to 72%) and without (0.6% to 20%) genital HPV detection during pregnancy (Watts *et al.* 1998). Infants exposed perinatally to HPV-11, or less commonly to HPV-6, may develop a rare benign tumor of the airway called juvenile-onset recurrent respiratory papillomatosis (Shoultz *et al.* 1997).

2.4.1 Acquisition and incidence

Only a few studies have estimated the incidence of genital HPV infection in the United States. An investigation of the general population of Rochester, MN, showed that the average age- and gender-adjusted incidence of genital warts increased from 13 per 100,000 in the early 1950s to 106 per 100,000 in the late 1970s. This time period corresponds to the years when rates for other sexually transmitted diseases increased dramatically in the United States (IARC 1995, Shoultz *et al.* 1997). The acquisition rate for HPV DNA appears to be much higher than that for genital warts, especially in younger women. Two prospective studies of young women in the United States who were HPV-negative at enrollment reported a somewhat similar 3-year cumulative incidence (43% and 55%) for acquiring any HPV infection (as measured by HPV DNA) (Ho *et al.* 1998a, Woodman *et al.* 2001). The 3-year cumulative incidence was 44% in a study from the United Kingdom (Woodman *et al.* 2001). A somewhat similar cumulative incidence rate (38%) was reported in a Brazilian study; that study included women aged 18 to 60, and the rate was over 18 months. In the Brazilian study, the incidence rate of HPV oncogenic types was higher in women under 35 (8.8/1000 women-months) than in those 35 years old or older (4.0/1,000 women-months), whereas the incidence rate of HPV nononcogenic types was relatively similar in both age groups (9.0/1,000 women-months [< 35 years old] vs. 9.2/1,000 women-months [≥ 35 years old]) (Franco *et al.* 1999).

2.4.2 Clearance

Longitudinal studies among cohorts of HPV-positive women have consistently reported an estimated 70% clearance rate after 12 to 24 months of follow-up (Dillner 2000, Franco *et al.* 1999, Moscicki *et al.* 1998) and a median duration of about 8 months (Liaw *et al.* 2001, Elfgren *et al.* 2000, Franco *et al.* 1999, Ho *et al.* 1998a). Some studies also have reported that low-risk HPV types were more likely to regress than high-risk types (Franco *et al.* 1999, Ho *et al.* 1998a, Moscicki *et al.* 1998, Elfgren *et al.* 2000). The median retention time of HPV positivity (time for 50% of the subjects to lose HPV positivity after enrollment) was significantly higher for high-risk types (8.1 months, 95% CI = 7.8 to 8.3) than for low-risk types (4.8 months, 95% CI = 3.9 to 5.6) in a cohort study of Brazilian women (aged 18 to 60) (Franco *et al.* 1999). In a 5-year follow-up study of HPV infection, type persistence (from first to fifth year) was observed in only 4 of 49 women; all 4 women with type persistence were infected with HPV-16 (Elfgren *et al.*

2000). Besides high-risk HPV, risk factors for persistent HPV infection include older age and multiple HPV types found at previous visits (Ho *et al.* 1998a).

2.5 Treatment

Currently, no antiviral treatments are available. Treatment of HPV infections depends on the severity of infection. Most low-grade infections regress spontaneously, although sometimes patients will seek treatment. Treatments of warts include topical applications, surgical excision, and interferon-related therapies. Higher-grade cervical lesions, such as CIN II or III, are usually excised by surgical procedures, cryotherapy, or laser methods. Treatment of cervical cancer depends on the extent of the lesion, the patient's age, and fertility considerations. Most patients undergo a hysterectomy, but conization may be done when the margins are free of disease. Treatment also may involve chemotherapy or radiation (Franco *et al.* 2001, Lowy and Howley 2001).

2.6 Immunity

The immune system plays an important role in HPV infections; immunocompromised patients are at an increased risk for persistent HPV infections. Humoral immunity is important in preventing the spread of infection to new sites within the host, whereas cellular immunity is more important for virus clearance from an infected host. As mentioned in Section 2.4.1, most HPV-associated cervical lesions regress; this regression of lesions is associated with a mononuclear cell infiltrate and may involve cellular immune response to E1, E2, E6, E7 and L2 proteins (Lowy and Howley 2001).

Protective immunity has not yet been elucidated but appears to involve both B- and T-cell immunity and to be type specific (Lowy and Howley 2001). Human leukocyte antigens (HLA) probably play a role in the immune response; HPV viral peptides or proteins are presented to T cells after being processed by Class I and II pathways (Cornelison 2000). Some studies (discussed in Section 3) have suggested that HLA haplotypes may modify HPV cancer risk (Lowy and Howley 2001).

2.7 Vaccines

Two types of vaccines are currently being developed: therapeutic vaccines, which target HPV-infected cells through cellular immunity components; and prophylactic vaccines, which are designed to prevent HPV infections by inducing neutralizing antibodies. As discussed above, cellular responses involving the E6 and E7 proteins are important in the regression of lesions; thus these proteins are targets for therapeutic vaccines. Neutralizing antibodies generally act against capsid proteins (L1 and L2); thus these antigens are usually employed for prophylactic vaccines. Effective vaccines should stimulate immune responses in mucosal and associated lymph nodes, and these responses may be best achieved by strategies that elicit both antibody and T-cell responses. Current vaccine models include peptide-based models, viral-like particles vaccine, DNA vaccines, and viral vector vaccines (Cornelison 2000). Early-phase clinical trials of prophylactic vaccines are being conducted using HPV-16 L1 capsid (VLP) antigens (Harro *et al.* 2001). Koutsky *et al.* (2002) recently reported promising results from a double-blind multi-center clinical trial using a HPV-16 L-1 virus-like-particle (L1 antigen without

DNA) vaccine. A study group of 2,392 young women were randomly assigned to receive placebo or the vaccine and followed for a median of 17.4 months. The incidence of persistent HPV-16 infection was 3.8 per 100 woman-years at risk in the placebo group compared to 0 per 100 woman-years at risk in the vaccine group. All nine cases of HPV-16-related CIN occurred in the placebo group, suggesting that immunization may reduce cervical cancer risks.

2.8 Regulations

There are no regulations specific for HPV. However, the U.S. Department of Health and Human Services has established regulations designed to ensure the reliability of Pap smear screening (42 CFR 493.1257). These regulations require laboratories that process and read Pap smears to adhere to specific regulations such as limiting the number of slides that may be screened in one day and ensuring the qualifications of the personnel at the laboratories.

2.9 Summary

HPV is one of the most common sexually transmitted infections, worldwide. More than two million people per year in the United States develop cytologic abnormalities of the genital tract that are caused by HPV infections. The major risk factors for HPV infection are behaviors associated with sexual activity. Thus, the highest prevalence rates (25% to 40%) are found among young, sexually active individuals. Although most infections are asymptomatic and are resolved spontaneously, HPV infection can lead to clinical disease, including anogenital warts, cervical neoplasia, cervical cancer, and other anogenital cancers. Diagnosis of HPV infections is made by either conventional clinical methods (e.g., by detecting visible lesions or by microscopic cytology) or by molecular diagnosis (e.g., DNA-based methods). Human epidemiological studies typically assess HPV infection by molecular diagnosis (HPV DNA) or serological responses. Treatment of HPV infections depends on the severity of the disease and includes topical applications, interferon-related therapies, laser methods, and surgical excision. Early-phase clinical trials of prophylactic vaccines are currently being conducted using HPV-16 L1 capsid antigens.

3 Human Cancer Studies

The carcinogenicity of HPVs was reviewed by IARC in 1995. IARC found compelling epidemiological evidence that some HPVs are human carcinogens and classified HPV types 16 and 18 as known human carcinogens (Group 1), HPV types 31 and 33 as probably carcinogenic to humans (Group 2A), and some HPV types other than 16, 18, 31, and 33 as possibly carcinogenic to humans (Group 2B). IARC also noted that some evidence suggested that HPV types 6 and 11 were not carcinogenic in the cervix of women. IARC based its conclusions on human studies of cervical cancer (IARC 1995). Since the 1995 review, a plethora of published studies has evaluated the role of HPV in the etiology of cancer in the cervix and other tumor sites. These studies have provided further elucidation of issues important in HPV carcinogenesis.

Because of the importance of cervical cancer in the review of HPV carcinogenicity, this section will briefly discuss cervical intraepithelial neoplasia, including progression to invasive cervical cancer. The review of human cancer studies consists of a brief discussion of IARC's evaluation and a more extensive compilation of the recent literature. Because of the numerous epidemiological studies published since 1995, this section does not attempt to identify and review every study, but rather summarizes many studies that are representative of the literature. This section focuses on the following types of studies or issues: (1) case-control studies reporting risk estimates for individual high-risk viruses (16, 18, 31, 33, 35, 39, 45, 51, 52, 58, 59), (2) cohort studies demonstrating that HPV infection occurs before cancer development, (3) studies of cancer at sites other than the cervix, (4) viral factors important in carcinogenicity including viral sequence variants and viral persistence, (5) cofactors and genetic factors important in HPV-induced carcinogenicity, and (6) evidence for causality.

3.1 Cervical intraepithelial neoplasia

Worldwide, approximately 500,000 new cases of cervical cancer are diagnosed each year. Both incidence and death rates vary geographically; the annual incidence rate (new cases per 100,000 women) ranges from 70 in Recife, Brazil and Cali, Colombia to less than 5 in Finland, Pakistan, and China (Garnett and Waddell 2000, Schoell *et al.* 1999). The death rate (deaths per 100,000 women) ranges from 21.3 in southern Africa to 3.4 in North America. Incidence rates in the United States vary according to race or ethnic group and have shown a general decline over the past decade. The average incidence rates in the United States from 1992 to 1999 were 9.6 in Whites, 13.3 in Blacks, 6.9 in American Indians/Alaska Natives, 11.7 in Asians or Pacific Islanders, and 17 in Hispanics (SEER 2002). Differences in disease incidence and mortality may be the result of prevention practices, such as the availability of screening and treatment programs (Garnett and Waddell 2000). Deaths from cervical cancer in the United States decreased by 74% between 1955 and 1992 as a direct result of the use of the Pap test in routine gynecological screening and the treatment of early disease detected in the screening program (American Cancer Society 2000, as reported in Garnett and Waddell 2000). In 1998, there were an estimated 4,900 deaths from cervical cancer in the United States (Schoell *et al.* 1999).

Cervical cancer is associated with several aspects of sexual behavior, including multiple sexual partners and early age at first intercourse. These observations led early investigators to evaluate sexually transmitted etiological agents including gonorrhea, syphilis, herpes simplex virus type 2 (HSV-2), and HPV (IARC 1995). Other risk factors include cigarette smoking, oral contraceptive use, increased parity, and maternal use of diethylstilbestrol (DES) (reviewed in Tjiong *et al.* 2001, Hatch *et al.* 2001).

3.1.1 Histological lesions associated with HPV infections: nomenclature

Invasive cancer of the cervix is preceded by precursor lesions. The term "cervical intraepithelial neoplasia" (CIN) originally referred to the spectrum of cervical diseases, beginning with mild dysplasia and ending with *in situ* carcinoma (CIN I, CIN II, and CIN III). After the accumulation of molecular data indicated that the changes were related to HPV infection, the classification was modified to a two-tier system consisting of low-grade (HPV infection) and high-grade (cancer precursor or precancer) CIN lesions. In 1989, a working group (National Cancer Institute Workshop 1989) established "The Bethesda System," which used the terms atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), and high-grade squamous intraepithelial lesion (HSIL), to provide a biologically meaningful classification of cervical disease and to clarify discrepancies of intra-observer and inter-observer reproducibility in cytological diagnoses. LSIL includes cytologic evidence of HPV infection with such names as CIN I or mild dysplasia, koilocytosis, koilocytic atypia, and flat condyloma; HSIL includes CIN II and especially CIN III or moderate and severe dysplasia and carcinoma *in situ* (IARC 1995) (Table 3-1).

Table 3-1. Cervical cytology: nomenclature

Bethesda System	Description	Cervical intraepithelial neoplasia
ASCUS: Atypical squamous cells of undetermined significance	Squamous atypia	
LSIL: Low-grade squamous intraepithelial lesions	Mild dysplasia, koilocytic or condylomatous atypia	CIN I
HSIL: High-grade squamous intraepithelial lesions	Moderate to severe dysplasia, carcinoma *in situ*	CIN II CIN III

Adapted from Jastreboff and Cymet 2002.

3.1.2 Natural history of CIN progression

The natural history of CIN progression is depicted in Figure 3-1.

The cervix consists of two histological components: the endocervix, composed of columnar cells, and the exocervix, composed of squamous cells. These components meet in an area defined as the transformation zone. Starting at puberty, the columnar cells slowly transform into squamous cells at the transformation zone. Cervical dysplasia most often occurs in the transformation zone (Jastreboff and Cymet 2002). Normal cervical

epithelium is characterized by an orderly arrangement (differentiating from basal layer to surface layer) of squamous epithelium, whereas HPV-related CIN is characterized by less organized growth patterns (Nuovo 2000).

HPV infects the cervix, causing visible acetowhitening lesions of varying degrees of severity, including warts, low- and high-grade CIN, and invasive cervical cancer (Einstein and Burk 2001). Low-grade CINs (CIN I or low-grade SIL; see Table 3-1) are well-differentiated lesions with alterations that are characteristic of the cytopathogenic effects of a replicative viral infection, such as binucleation, perinuclear cytoplasmic cavitation, and nuclear atypia; they are usually not considered to be true cancer precursors. The majority of CIN I lesions are transient and usually resolve spontaneously, but approximately 1% progress to invasive cancer (Jastreboff and Cymet 2002). Both high-risk and low-risk HPVs can cause low-grade SIL, and the lesions caused by different viral types are indistinguishable by light microscopy (IARC 1995). The prevalence of low-grade SIL decreases with increasing age (Adam *et al.* 2000, Herrero *et al.* 2000).

High-grade CIN lesions are characterized by undifferentiated cells that extend past the lower third of the epithelium and by nuclear crowding, substantial pleomorphism, loss of tissue organization and cellular polarity, abnormal mitotic figures, and greater atypical cytology than low-grade CIN (IARC 1995). High-grade CIN lesions probably result from persistent HPV infections. No cases of high-grade SIL were observed in a longitudinal study of the natural history of HPV infection among individuals whose infection had cleared by a third visit (Schlecht *et al.* 2001); however, another study reported the appearance of high-grade CIN without prolonged HPV infection (Woodman *et al.* 2001).

The prevalence of high-grade lesions appears to increase with age up to 30 to 40 years (Herrero *et al.* 2000, Adam *et al.* 2000), then decreases in women over 40. High-grade lesions may develop from low-grade CIN but they can also develop without any evidence of low-grade lesions. CIN lesions regress in about 80% of women less than 34 years old and in about 40% of older women (Herrero and Muñoz 1999). Approximately 5% of CIN II progress to invasive cancer (Jastreboff and Cymet 2002). Left untreated, a significant number of CIN III cases will progress to invasive cancer (Garnett and Waddell 2000). Treatment of CIN II and CIN III is the same; therefore, they are grouped together clinically (Jastreboff and Cymet 2002). However, in the Manchester cohort study, the prevalence of CIN III but not CIN II was reported to increase linearly with increasing time since the last normal Pap smear, suggesting that some cases of CIN II may not be equivalent to high-grade SIL (precancer) (reviewed by Bosch *et al.* 2001).

Microinvasive squamous-cell cancer usually arises from high-grade CIN. It is characterized by single or multiple irregular tongues of neoplastic squamous epithelium, penetration through the plane of the basal lamina, and invasion of the cervical stroma or epithelial lamina propria. Microinvasive foci and invasive carcinoma have similar histological appearances but differ in the degree to which they invade the cervical stroma (3 mm or less for microinvasive foci and greater than 3 mm for invasive cancer).

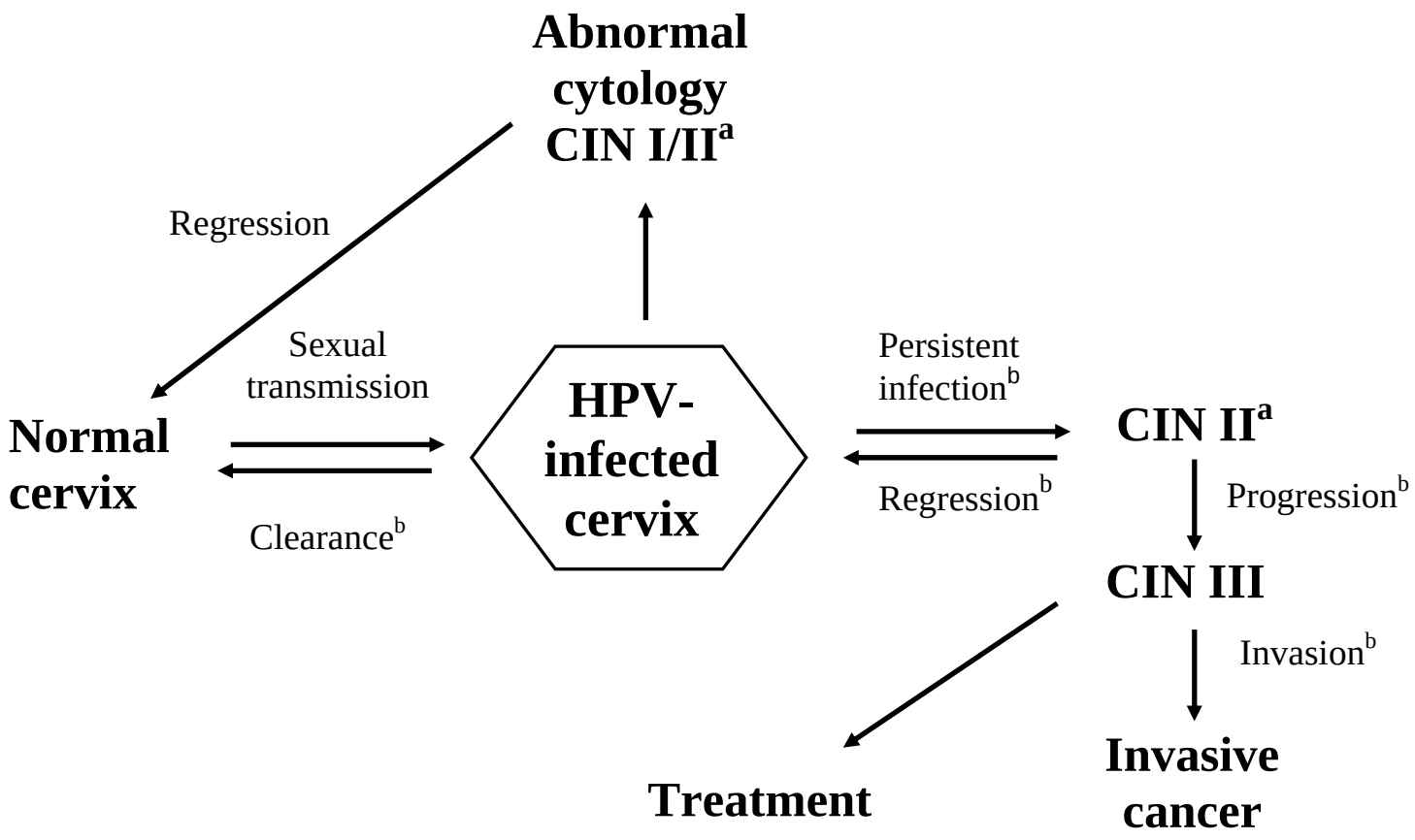


Figure 3-1. Natural history of CIN progression

[a]CIN II is an intermediate group defined by histopathology, but it is heterogenous in viral typing and natural history; most CIN II cases are acute and regress

[b]Cofactors: Genetic, immune system, hormones, smoking

3.1.3 Other anogenital cancers related to HPV infection

Adenocarcinomas *in situ* and adenocarcinomas occur in the columnar cells of the cervix and are characterized by complex gland formation, cytological atypia, increased mitotic rate, and gland-within-gland patterns. Both lesions are associated with CIN, especially high-grade lesions (IARC 1995).

HPV infection induces histological changes in the vagina similar to those in the cervix, and squamous neoplasms of the vagina and anus are similar in morphology to those of the cervix (IARC 1995). Three types of intraepithelial lesions occur in the vulva (VIN): basaloid, warty, and well differentiated. Basaloid VIN are composed of small uniform cells that are hyperchromatic and contain alterations in the nuclear chromatin distribution patterns; warty type VIN are highly pleomorphic lesions containing multinucleated cells and are characterized by chromatin clumping, cytological atypia, and a large number of mitoses. High-risk HPVs are found in both of these types but rarely in well-differentiated VIN, which are proliferative lesions with minimal nuclear atypia and alteration of pattern (IARC 1995).

3.2 IARC evaluation

In a comprehensive and detailed evaluation of HPV carcinogenicity, IARC reviewed case-report and case-series studies, cohort studies, and case-control studies of cervical cancer, other anogenital cancers (vulvar/vaginal, penile, and anal), and other cancers. IARC also discussed studies evaluating cofactors important in HPV-associated carcinogenicity and studies in special populations including renal-transplant patients, HIV-infected individuals, and patients with epidermodysplasia verruciformis (IARC 1995).

IARC concluded from their evaluation of numerous case-series studies that HPV was found in a high proportion of cervical cancers. The majority of case-series studies (which had over 40 cases) detected HPV in greater than 75% of cases with squamous cell carcinoma (SCC) of the cervix (range 57% to 100%); HPV DNA was detected in 93% of the cases in the largest study. HPV-16 was the most prevalent HPV type. HPV was detected in 15% to 88% of case-series studies of adenocarcinoma and adenosquamous carcinoma (ADS) using hybridization detection methods, with HPV-16 and 18 the predominant types. Although overall HPV prevalence varied little geographically, there were geographical differences in the distribution of types. HPV was also detected but with considerable study-to-study variation in noncervical anogenital cancers and other cancers; fewer studies were reported for nonanogenital cancers (IARC 1995).

Most of the prospective studies of HPV infection and cancer reported by IARC focused on the cervix and consisted of two types of cohort studies: (1) transition from HPV infection to the development of cervical intraepithelial neoplasia (CIN) and (2) progression of CIN to cancer or high-grade CIN. Most of these studies demonstrated that HPV-positive women (assessed by HPV DNA) had a higher risk for both development and progression of CIN, demonstrating that HPV infection precedes the development of

high-grade CIN. Studies evaluating different HPV viral types found HPV-16 or 18 but not 6 or 11 to be associated with CIN progression (IARC 1995).

The IARC monograph provides a comprehensive listing and discussion of numerous (~100) case-control studies of cervical carcinoma. These are organized by both CIN stage (CIN I-III and invasive carcinoma) and HPV detection method (HPV DNA by nonhybridization methods, hybridization methods without target amplification, hybridization assays including PCR amplification, and serological assays). However, a limited number of studies that met criteria related to sound epidemiological study design, state-of-the-art exposure assessment (PCR methods), and use of multivariate statistical analyses were the most influential in the Working Group's final evaluation of causality. Case-control studies of the highest quality, which measured the association of cervical neoplasia and HPV DNA, consistently reported strong odds ratios (ORs) that were usually > 10 (in fact, most were > 20) among the most informative studies (Figure 3-2). The association was specific to a limited number of viral types, e.g., 16, 18, 31, 33, and 45, which are the most prevalent types found in cervical cancer biopsies. Case-control studies measuring HPV antibodies (mainly to E6 and E7) were consistent with the findings from studies based on HPV DNA detection; however, in general, the magnitude of the risk estimates was lower (IARC 1995).

The IARC monograph also evaluated the association of HPV with other anogenital cancers and other cancers; the literature for most of these sites consisted of only a few studies. HPV infection (assessed by genital warts or HPV DNA detection) was associated (ORs from 2.8 to ∞) with other anogenital cancers (penile, anorectal, and vulvar) and some nonanogenital cancers (including oral cancer and cancers of the upper digestive and respiratory tract) (IARC 1995).

IARC recognized that cofactors are probably important in HPV-induced carcinogenicity because of the high prevalence of HPV infections compared to the relative rarity of anogenital cancers. Nevertheless, IARC also noted that the large magnitude of the relative risks between HPV infection and cervical cancer could not be explained by confounding or effect modification from other factors. Possible cofactors include hormonal (as inferred from parity and oral contraceptive use) and immunological variables (including HLA genotypes and immunosuppression). The roles of other infectious agents and smoking were unclear (IARC 1995).

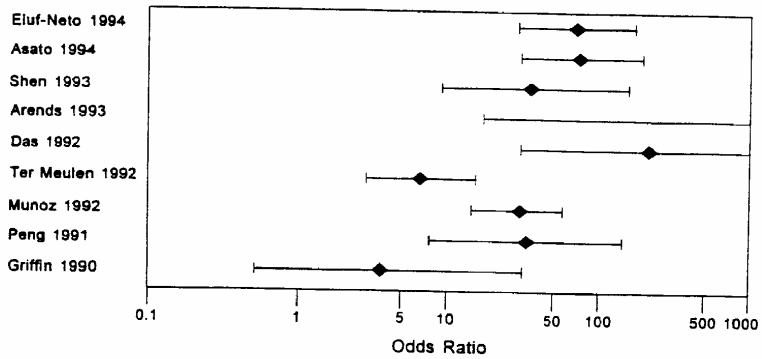


Figure 3-2. Odds ratios and 95% confidence intervals for associations between HPV-16 and invasive cervical cancer found in case-control studies that used PCR methods and were published before the 1995 IARC evaluation

IARC also reviewed human cancer studies in three special populations: patients with EV, transplant patients, and HIV-infected individuals. EV is a rare inherited condition characterized by widespread HPV infections manifested by skin warts. HPV types 5, 8, 14, 17, and 20 have been identified in EV-associated squamous-cell carcinomas, although only in a small number of tumors (these HPV types are not included in the present review). The IARC publication noted that several studies reported increased rates of HPV infection among transplant recipients; other studies reported a higher risk of CIN, invasive cervical cancer, and other anogenital cancers in transplant patients. Several studies also have reported that HPV is more prevalent in HIV-infected individuals, measured in precancerous lesions from both women (CIN) and men (anorectal); and a few studies demonstrated that the prevalence of HPV increased with the degree of immunosuppression as assessed by decreasing numbers of CD4+ cells. HIV and the degree of immunosuppression were associated with CIN progression but not with increased risk of invasive carcinoma, which may be partly explained by the late introduction of HIV into the female population (IARC 1995).

IARC concluded that compelling evidence exists that some HPV types are human carcinogens, based on human studies of cervical cancer. Their evaluations were as follows (IARC 1995):

- The strongest evidence was for HPV-16 and cervical cancer, where numerous case-control and some prospective studies were available.

- A causal role for HPV-16 in anal cancer was highly likely even though no prospective studies were available. The epidemiological data were limited for HPV-16 and other anogenital cancers (vulvar and penile) and inadequate for cancers at other sites.

- The carcinogenicity evidence for HPV-18 was limited to the cervix, where HPV was associated with a minority of squamous cell cancers and approximately half of the adenocarcinomas.

- Other HPV types were implicated as human carcinogens, the strongest evidence being for HPV-31 and 33; evaluation of these types was limited by the low prevalence for individual specific types.

- The data suggested that HPV-6 and 11 were not human carcinogens for the cervix; data at other sites were inadequate

3.3 Human cancer studies since the IARC evaluation

This section reviews the most informative studies on cervical cancer (organized by study design), other anogenital cancers, and head- and neck-related cancers. These studies assessed HPV exposure either by measuring HPV DNA or by HPV serology (see Section 2). Because most studies detected HPV DNA by PCR methodology using consensus primers, the detection methodology used in each study is not described below but is presented in the tables in Appendix A. For studies not detecting HPV DNA by PCR methodology, the detection methods are noted in the study descriptions. Most detection methods used in these studies can type individual viruses; however, many studies calculated only pooled ORs for a group of viruses (e.g., high risk, or HPV-16 related) rather than for individual virus types, probably because of the small number of cases and controls that have a specific virus.

3.3.1 Cervical cancer

3.3.1.1 Case-series studies

The most recent studies of invasive cervical cancer using sensitive detection methods indicate that HPV is present in virtually all cervical carcinomas, suggesting that HPV may be a necessary cause of cervical cancer (Walboomers *et al.* 1999, Bosch *et al.* 1995, van Muyden *et al.* 1999). Earlier studies that reported lower prevalence estimates were probably compromised by suboptimal tissue specimens and less sensitive detection assays. The International Biological Study of Cervical Cancer, which collected more than 1,000 tissues from invasive cervical cancers in women from 22 countries, reported an HPV DNA prevalence estimate of 93% based on a PCR assay using MY09/MY11 primers (Walboomers *et al.* 1999, Bosch *et al.* 1995). Walboomers *et al.* (1999) reanalyzed the HPV-negative samples from this study using two PCR assays, E7 primers and consensus primers. The two assays (combined) detected HPV in 73% of the original biopsies that had been HPV-negative and for which sample were available that had DNA adequate for PCR amplification (55 of the original 66 HPV-negative biopsies). The authors hypothesized that HPV may not have been detected originally in some of the samples because the MY09/11 primers failed to amplify integrated HPV genomes. The worldwide HPV prevalence in cervical carcinomas estimated from the combined data of these studies is 99.7% (Walboomers *et al.* 1999).

The International Biological Study of Cervical Cancer detected HPV-16 in 50% of the samples, HPV-18 in 14%, HPV-45 in 8% and HPV-31 in 5%. Sixty-seven percent of the viruses detected were members of the HPV-16 phylogenetic group (i.e., 16, 31, 33, 35, 52, and 58), and 27% were members of the HPV-18-related group (i.e., 18, 39, 45, 59, and 68). HPV-16 was the predominant type in almost all countries (except Indonesia). The prevalence of other viral types varied geographically; HPV-45 was more common in western Africa, while HPV-39 and 59 were detected mainly in Central and South America. The prevalence of virus type also varied by histological types. HPV-16-related viruses predominated in SCCs (52% of the tumors), whereas HPV-18-related viruses were predominant in adenocarcinomas (56% of the tumors) and adenosquamous carcinomas (39%); however, there were few cases of adenocarcinomas (n = 24) or adenosquamous carcinomas (n = 17) (Bosch *et al.* 1995).

3.3.1.2 Case-control and cross-sectional studies

Case-control and cross-sectional studies of cervical precancer (CIN III) and cancer published since the 1995 IARC review are described in Table A-1 (Appendix A). These studies assessed HPV exposure by measuring either HPV DNA or by HPV serology (see Section 2). Some studies reported risk estimates for multiple stages of CIN, including cancer; others only reported risk estimates for invasive cancer. The following sections discuss risk estimates for precancer (CIN III, carcinoma *in situ*, or high grade SIL) or cancer (unless only a combined estimate for many stages of CIN is given) and are organized by endpoint and exposure measurement.

3.3.1.2.1 Studies of precancer (CIN) or invasive cancer measuring HPV DNA

Five studies (United States, Honduras, China, Costa Rica, and Japan) evaluated the role of multiple HPV viruses in women with various diagnoses of CIN and cancer. Most of these studies reported risk estimates for groups of viruses, e.g. high-risk, HPV-16, or 18-related viruses, rather than for individual viruses, probably due to small numbers of individual positives for each specific virus. The study populations in these studies came from either a population- or hospital-based screening program. Women who had cervical abnormalities at the screening became the cases, and women without cervical abnormalities became the controls. HPV was assessed by measuring and typing HPV DNA.

Lorincz *et al.* (1992) calculated risk estimates for specific HPV types (as assessed by detection of HPV DNA measured by Southern hybridization, which is not as sensitive as PCR-based methods) and cervical neoplasia (invasive cancer, and high- and low-grade SIL) using data from 2,627 women who participated in eight studies (cohort, case-series, and case-control) in various places in the United States. Risk estimates for invasive cancer were highest for HPV-16 (OR = 260) and the HPV group consisting of types 18, 45, and 56 (OR = 296), intermediate for the HPV group consisting of 31, 33, 35, 51, and 52 (OR = 31), and lowest (OR = 0) for the HPV group consisting of HPV-6, 11, 42, 43, and 44. Based on the results, these virus types were classified as high, intermediate, and low risk, respectively. Risk estimates for intermediate-risk viruses were higher for high-grade SIL than for invasive cancer. Most recent studies include intermediate types (31, 33, 35, 51, 52, and 58) in the high-risk category. Although the study reported here was

published before the IARC evaluation, it is discussed in the current studies section because of its key role in defining high-risk, intermediate-risk, and low-risk viruses.

The Chinese study included 332 women from Hong Kong who were diagnosed at a hospital colposcopy clinic (Chan *et al.* 1999). Cases included 158 women with cervical abnormalities ranging from CIN I to invasive cancer. Controls included 174 women with normal cervixes, inflamed cervixes, or condyloma. HPV-58, which is uncommon worldwide, was the second most common (after HPV-16) viral type found in the women with cervical neoplasia. Both HPV-16 (OR = 7.4) and HPV-58 (OR = 3.7) were significantly associated with CIN (CIN I to CIN III) and carcinoma (combined), and the prevalence of both viral types significantly increased with increasing severity of cervical lesions. Risk estimates were probably lower than in other studies because CIN I and II cases were included.

Ferrera *et al.* (1999a) conducted a population-based case-control study of 229 women with CIN and invasive cancer and 438 women with normal cytology from hospitals and clinics in Honduras. Elevated ORs for invasive cancer (ORs ranged from 13 to 24) and CIN III (ORs ranged from 24 to 48) were observed for HPV-16-related viruses (16, 31, 33, 35, 52, 58) and HPV-18-related viruses (18, 45, 59). Other HPV viruses were detected at low frequencies. Risk estimates for high-risk HPV types were lower than those reported by Lorincz *et al.* (1992), which may indicate differences in the viral composition of the viral group (HPV-16 and 18-related versus HPV-16, other high risk, and intermediate risk), detection assay (PCR versus Southern blot analysis), geographical location (Honduras versus multiple centers in the U.S.) or, most important, differences in the prevalence of HPV in controls (39%, Honduras; 6.4%, Lorincz's study).

The Costa Rican study population consisted of a subset of women (n = 2,974), who were tested for more than 40 HPV types and for cervical abnormalities, from a large-census cohort of 9,175 women (Herrero *et al.* 2000). Risks were calculated for low-grade and high-grade SIL and for cervical cancer. Very high risk estimates were found for HPV-16 and high-grade SIL (RR = 320) and cervical cancer (RR = 710). Risk estimates were lower for other high-risk viruses (HPV-18, 31, and 45) and for a separate group consisting of other cancer types, i.e., high-risk, but the latter were still of considerable magnitude. In contrast to Lorincz's study, risk estimates for other cancer types, which probably include viruses that Lorincz *et al.* (1992) grouped as intermediate, were higher for invasive cancer than for high-grade SIL; however, the makeup of the virus groups in each study was not identical.

Sasagawa *et al.* (2001) calculated risk estimates for multiple HPV viruses in 366 women with various cervical lesions, including high- and low-grade SIL and invasive cancer, and for 11,562 women without CIN. The study population came from a hospital screening program in Japan in which women were diagnosed for cervical abnormalities at enrollment. Based on these risk estimates, the authors concluded that HPV-6 and 11 were low-risk types, and 16, 18, 31, 51, 52, 58, and perhaps 33, 35, 45, 56, and 67 were high-risk types in Japan. Many HPV types were associated with significant risk for low-grade SIL. Multiple infections were associated with all types of lesions (low- and high-grade

SIL and invasive cancer), but the association decreased with increasing severity of the lesions.

3.3.1.2.2 Studies of invasive cancer measuring HPV DNA

Several case-control studies, including one from Thailand and a series of IARC-initiated studies, have reported risk estimates for HPVs and invasive cancer based on detection of HPV DNA using PCR-based assays. The study from Thailand reported estimates for HPV-16 and 18 and for groups of HPV types, whereas the IARC-initiated studies reported estimates for individual types.

Thomas *et al.* (2001a) conducted a hospital-based case-control study in Bangkok which consisted of 190 cases of SCC, 42 cases of adenocarcinoma (ADC), and 291 hospital controls. Several high-risk HPV types (HPV-16 and 18, and the combined group 31, 33, 35, 39) were strongly associated with both squamous cell and adenomatous carcinoma. Risk estimates (ORs) for any oncogenic type (i.e., 16, 18, 31, 33, 35, 39, and 45) were 155 (95% CI = 72 to 385) for squamous cell and 106 (95% CI = 41 to 317) for adenomatous carcinoma; risk estimates for any HPV type were 97 (95% CI = 52 to 193) for squamous cell and 49 (95% CI = 21 to 125) for adenomatous carcinoma. In a related study, cases of invasive carcinoma also were compared to cases of carcinoma *in situ* (identified at the same time as the invasive cancer cases to determine progression of CIN lesions). Significantly elevated risks for progression were observed for HPV types 16 and 18, but lower, nonsignificant risks were found for the combined (31, 33, 35, 39) HPV viral types (Thomas *et al.* 2001b).

IARC conducted a series of 13 multicenter, case-control studies on invasive cervical cancer in various geographical locations to assess the risk associated with specific HPV types and to evaluate the role of cofactors. Two publications on five studies (one publication of two studies from Spain and two from Colombia and one publication on a study from Brazil) were available for the 1995 IARC review. [ORs were only reported for HPV-16 and 18, the HPV group of 31, 33, and 35, and undefined HPVs]. Results from three studies have not yet been published, although the data from one study (Mali) have been used in a pooled analysis reported in a recent review article (Muñoz 2000). The five studies that have been published since the IARC review (Morocco (Chaouki *et al.* 1998), Thailand (Chichareon *et al.* 1998), the Philippines (Ngelangel *et al.* 1998), Paraguay (Rolón *et al.* 2000), and Peru (Santos *et al.* 2001)) are methodologically similar with respect to study design, detection of exposure, and statistical analysis. These are all hospital-based studies, consisting of cases (ranging from 116 cases from Paraguay to 377 cases from Thailand) with incident invasive cervical cancer (both SCC and adenocarcinoma/adenosquamous carcinomas) and age-matched controls recruited from the same or nearby hospitals and without a history of hysterectomy or diagnosis related to established or suspected risk factors of cervical cancer. HPV DNA was detected and typed using a sensitive PCR assay (GP5+/GP6+ consensus primers; see Section 2.1.3.2). Study participants also were interviewed using a detailed questionnaire on factors related to cervical cancer risk.

High-risk HPVs were strongly associated (ORs often greater than 100) with invasive cervical carcinoma (SCC and adenocarcinoma/adenosquamous carcinomas); however,

geographical regions varied in the strength of positive associations detected for different HPV viral types. Positive associations were observed for HPV-16, 18, and 45 in all studies; for HPV-31, 33, and 58 in four of the studies (HPV-31 and 33 for Morocco [OR combined for 31, 33, and 35], Paraguay, Thailand, and Peru; and HPV-58 for Thailand, the Philippines, Paraguay, and Peru) for HPV-52 in three of the studies (the Philippines, Thailand, and Peru); and for other HPVs (HPV-35, 39, 51, 50s combined [51, 52, 56, 58, and 59], and 66) in studies from Morocco (HPV-50s combined), the Philippines (HPV-51 and 66) and Peru (HPV-35 and 39). One concern about these studies was that risk estimates might have been biased due to differences in the types of samples used to ascertain exposures; DNA was isolated from biopsies in cases but from exfoliated cells in controls. This could potentially underestimate the prevalence of HPV DNA in controls. However, a validation study found that the prevalence estimates of HPV DNA were identical from both types of samples (exfoliated cells and paired biopsy specimens) among women with normal cervixes undergoing hysterectomy for reasons other than cancer (de Sanjosé *et al.* 1999) cited in Rolón *et al.* (2000).

3.3.1.2.3 Studies of invasive cancer measuring HPV serology

In general, seroepidemiological studies only assess a limited number of HPV viruses (HPV-16, -18 and sometimes 33), and risk estimates are much lower than those for HPV DNA detection.

Population-based case-control studies from Sweden (Wang *et al.* 1997) and the United States (Carter *et al.* 2001) evaluated HPV seropositivity and invasive cervical cancer. Both studies had adequate numbers of cases and controls, 243 cases in the Swedish study and 483 cases of SCC and 305 cases of ADC and adenocarcinoma *in situ* (ACIS) in the study from the United States, and both studies measured HPV-16 and HPV-18 seropositivity; the Swedish study also measured HPV-33. High-risk HPV seropositivity (ORs between 1.5 and 3.6) was significantly associated with invasive cancer in both studies. The study from the United States reported separate ORs for ADC/ACIS and SCC; for SCC, the risk was slightly higher for HPV-16 than for HPV-18, and the opposite pattern was observed for ADC/ACIS. HPV-33 seropositivity was no longer associated with cervical risk in a later reanalysis of the Swedish population (with a few additional cases) that excluded women seropositive for other HPV viral types (Silins *et al.* 1999). Stronger associations were observed for HPV seropositivity in a study restricted to women who had HPV-16 or 18 DNA-positive cancers (Carter *et al.* 2001).

3.3.1.3 Cohort and nested case-control studies

Current cohort studies and nested case-control studies, which follow women with normal cytology until the development of CIN or invasive cancer, are described in Table A-2 (Appendix A) and are organized according to measure of exposure (DNA or serology).

3.3.1.3.1 Cohort studies measuring HPV DNA

As discussed in Section 3.1, a significant number of untreated CIN III cases will develop into cervical cancer, and thus, CIN III is an appropriate endpoint for assessing cancer risk (Figure 3-1). The cohorts in most studies that measured HPV DNA were derived from women participating in a screening program; thus, most women would have been treated

before they developed invasive cancer. Some of the studies calculated a risk estimate for high-grade CIN or high-grade SIL, which includes CIN II cases and may be less informative for evaluating causality because most CIN II cases regress. This section includes discussion of three longitudinal cohort studies that followed women until the development of high-grade CIN (including CIN II), and six nested case-control studies (including two archival studies), in which the cases are women who developed high-grade CIN, CIN III (or carcinoma *in situ*), or invasive cancer (archival study). In general, these studies did not evaluate individual viruses except for HPV-16 or HPV-18, but they did calculate risk estimates for the high-risk HPV category.

Two longitudinal studies from England (Woodman *et al.* 2001) and the United States (Moscicki *et al.* 1998) evaluated the natural history of HPV infection leading to CIN development in young women who had recently become sexually active. The English study followed 1,074 women who were both HPV negative and cytologically normal at enrollment; the United States study followed 618 HPV-positive women who were cytologically normal. The purpose of the latter study was to evaluate HPV regression and the risk of developing SIL; the English research also evaluated the incubation curve for HPV-associated abnormality. In the English study, 407 women became HPV positive, 245 developed an abnormal smear, and 28 developed high-grade CIN (14 women each with CIN II and III). Elevated relative risks for high-grade CIN were observed for HPV-16, 18, 31, 52, 58, and 6 or 11 (one category), and a decreased risk was observed for HPV-33; however, these estimates were based on a small number of cases. Five women who progressed to high-grade CIN were negative for HPV. Risk estimates for high-grade SIL (n = 33) in the U.S. study depended on viral persistence, as measured by positivity at consecutive visits; risks were higher for those who were positive for high-risk HPV on at least three visits (OR = 14.1), somewhat lower for two visits (OR = 8.9) and only slightly elevated for positivity at only one visit (OR = 1.1) (see Section 3.4 for a discussion on persistence). HPV DNA in the U.S. study was detected by RNA-DNA dot blot hybridization.

A third longitudinal cohort study, the Ludwig-McGill cohort study (Schlecht *et al.* 2001), evaluated the development of high-grade SIL in 1,611 Brazilian women, aged 18 to 60, with normal cytology and HPV results for first two visits; 286 women were positive for HPV at enrollment. Women were followed every four months the first year and twice yearly afterwards. HPV detection (oncogenic and 16/18) was associated with high-grade SIL, and risks were higher for women having positive HPV detection on both visits (RR for HPV-16 or 18 = 12.3 for both visits versus 3.9 for one visit). Persistent HPV infection of oncogenic types (positive for both visits) other than 16 and 18 also was associated with high-grade SIL (RR = 9.7), whereas persistent HPV infection of nononcogenic types was not associated with high-grade SIL (RR = 0.0).

Nested case-control studies conducted in Oregon by Liaw *et al.* (1999), the Netherlands by Rozendaal *et al.* (2000), Sweden by Ylitalo *et al.* (1999), South Carolina by Coker *et al.* (2001), and from Utrecht, the Netherlands by van der Graaf *et al.* (2002) followed cohorts of women with no history or present evidence of cervical abnormalities (at enrollment) for the development of incident CIN (various grades in the two studies from the United States, CIN III in the Netherlands, carcinoma *in situ* in Sweden, and CIN III,

and invasive cancer in Utrecht). The Netherlands cohort (Ylitalo *et al.* 1999) also included women with borderline cytology. Cases were identified by cervical smears taken during the follow-up period (two U.S. studies and two studies in the Netherlands) or by cancer registry (Sweden). Controls, women who did not develop CIN, were matched to cases by age and other factors related to cohort enrollment (Sweden, Utrecht, and two U.S. studies) or randomly chosen from the cohort (the Netherlands). HPV DNA was measured from cervical smears taken at enrollment or at a follow-up exam but before the diagnosis of disease. The study from the Netherlands detected HPV DNA using a PCR-enzyme immunoassay (EIA) whereas the other studies detected HPV DNA by PCR methodology. All studies reported a positive association between high-risk HPV DNA detection (either as a group or for HPV-16 or 18 individually) and development of high grade CIN: CIN III (the Netherlands), carcinoma *in situ* (Sweden), and high-grade SIL (two U.S. studies). The Utrecht study reported that HPV-16; HPV types 18, 31, and 33 combined; high-risk HPV types; and low-risk HPV types all had a positive association with CIN III or invasive cancer. Risk estimates increased with increasing severity of cervical abnormality in the Portland (Oregon) study. A very high risk for CIN III (OR for high-risk HPV = 210) was observed in the Netherlands study, albeit the estimate was based on a small number of CIN III cases (n = 13). A very high risk estimate for HPV-16 (OR = 104) also was observed in the Utrecht study for CIN III and invasive cancer, based on a larger number of cases (n = 77). Risk estimates were lower in the other studies but still of considerable magnitude. ORs for carcinoma *in situ* in the Swedish study were 15.9 for HPV-16 and 2.3 for HPV-18. ORs for high-grade SIL were 64 (HPV-16) and 31 (other high-risk HPVs) in the Portland study and 3.8 (other high-risk HPVs in the South Carolina study). The ORs for high-grade SIL in the U.S. studies were based on small numbers of cases (47 in Portland and 22 in South Carolina), while the Swedish study had a large number of cases of carcinoma *in situ* (n = 469). In the Utrecht study, lower ORs for CIN III and invasive cancer (compared to HPV-16) were observed for high-risk HPV (24) and low-risk HPV (5.4). Low-risk HPV types included 6, 11, 34, 40, 42, 43, 44, 53, 54, 66, 70, and 74. The ORs for low-risk HPV were based on four cases, a second smear (between baseline and diagnosis) for two of these cases showed that the women also had developed a high-risk HPV type. Women who had multiple HPV types at enrollment had a higher risk (OR = 50.6) than women who had a single HPV type (OR = 15.8).

Two archival studies, from Sweden and the Netherlands, have been reported. Wallin *et al.* (1999) conducted an archival study consisting of women who participated in a population-based screening in Sweden and who had at least one cytologically normal Pap smear and at least one additional Pap smear that had been stored; these women had not undergone surgical treatment of the cervix. Cases (n = 118) of incident invasive carcinoma were identified by linkage between the cytology registry and Swedish cancer registry; controls, women who did not develop cervical cancer, were matched to cases by age and factors related to Pap smear collection. A total of 30 percent of cases had detectable HPV DNA at baseline compared to only 3 percent of the controls (OR = 16.4, 95% CI = 4.4 to 75.1). HPV DNA also was assessed in the biopsy specimen taken at diagnosis. HPV type-specific persistence (defined as detection of the same HPV type at baseline and diagnosis) substantially increased cancer risk (OR = 58.7). HPV-16 and 18 were detected in the majority of samples, although HPV-31, 33, and 73 also were detected in a few samples.

Zielinski *et al.* (2001) conducted a retrospective case-control study of 57 women who developed cervical cancer between 1991 and 1998. This study included 114 age-matched controls who had participated in a population-based cervical screening program started in Zeeland (a district in the Netherlands) in 1976 and whose first smear was taken between 1976 and 1996. In the initial study, the baseline smear of the 57 cases and 114 controls was reported to be normal; however, upon reassessment of the cytology (second study), only 10 cases and 104 baseline smears were considered normal. High-risk HPV was associated with cervical cancer in both the original (ORs = 18, 95% CI = 11 to 72) and the second study (ORs = 32, 95% CI = 6.8 to 153).

3.3.1.3.2 Cohort studies measuring serology

The studies discussed below include two on CIN III (or the equivalent), two on carcinoma *in situ* and invasive cancer (combined), and one on invasive cancer. In general, the studies that included cases of invasive cancer were not screening cohorts, and the cases were identified from cancer registries. The serological studies only evaluated a limited number of HPV viruses (HPV-16, 18, and 33).

HPV seropositivity was associated with CIN development in nested case-control studies in Sweden (Chua *et al.* 1996) and Prague, Czech Republic (Vonka *et al.* 1999). The Swedish study identified cases from a population cohort of more than 15,000 women blood donors by matching the cohort to a morphology registry of cervical smears after a mean follow-up of 35 months. Controls were women with a CIN diagnosis during the follow-up period and matched to cases for age, date of blood sampling, and residence. Women were not examined for cytology at enrollment. Risk estimates were only slightly above 1.0 for HPV-18 and 33 seropositivity but were higher and significant for HPV-16 seropositivity (OR = 3.0, 95% CI = 1.5 to 6.0, $P = 0.001$). Similar estimates for HPV-16 seropositivity (OR = 3.9) and higher but nonsignificant estimates for HPV-18 (OR = 2.7) and 33 seropositivity (OR = 1.5) were observed in the Prague study. The Prague study (Vonka *et al.* 1999) followed a cohort of more than 10,000 women with no history or present cytological evidence of CIN (ranging from moderate dysplasia to invasive carcinoma) for 2 years. Risk estimates were based on 67 cases who developed cervical neoplasia and 129 controls who had no pathology; these women were matched to cases for age, sexual behavior, smoking, and DKG (diathermo-electrocoagulation of the ectopic epithelium and transformation zone). Both studies were limited by the small number of cases (< 100).

Lehtinen *et al.* (1996) conducted a nested case-control study within the Finnish Social Insurance Institution cohort, which consisted of 18,814 women who were free of cancer and had donated blood. Cases of invasive carcinoma (n = 27) and carcinoma *in situ* (n = 45) were identified by the cancer registry; controls (n = 143), free of cancer, were matched to cases for age and residence municipality. HPV-16 capsid antibodies, measured from serum taken at enrollment, were associated with an elevated risk of invasive cancer (OR = 12.5). A similarly designed nested case-control study was conducted by Shah *et al.* (1997) in the United States. This study identified 14 incident cases of invasive carcinoma and 28 incident cases of carcinoma *in situ* by cancer registries and selected non-cancer controls, matched to cases by age and factors related to the collection of blood, from a population-based cohort of women who donated blood and

answered a brief questionnaire. Risk of disease was positively associated with HPV-16 but not with HPV-6 seropositivity. Moreover, the risk was higher in individuals who had higher antibody titers, as assessed by absorbance in an ELISA assay (ORs for low titer = 3.9 and for high titer = 7.5).

Dillner *et al.* (1997) conducted a nested case-control study whose participants were 182 cases who had invasive cancer and 538 matched controls from a study base from three population serum bank cohorts in Finland, Norway, and Sweden. Cases were identified from cancer registries. Elevated risks for HPV-16 (significant), HPV-18 (significant), and HPV-33 (not significant) seropositivity were reported after adjusting for other sexually transmitted diseases and smoking. In contrast to Lehtinen *et al.*'s (1996) study, Dillner *et al.* observed a lower OR for HPV-16 (OR = 2.2), and in contrast to Shah *et al.*'s (1997) study, the OR in Dillner's study did not increase with higher antibody titers. Most of the cases in the studies by Lehtinen *et al.* (1996) and Shah *et al.* (1997) had carcinoma *in situ*.

3.3.1.4 Longitudinal studies of CIN progression

CIN progression studies are described in Table A-3 (Appendix A). These studies assessed HPV exposure by measuring HPV DNA, and they reported risk estimates for groups of viruses rather than individual viruses.

High-risk HPV was associated with CIN progression in two follow-up studies, from the Netherlands and Brazil, of women with abnormal cytology. The Netherlands study followed a cohort of 342 women, 19 of whom developed CIN III (Remmink *et al.* 1995). In a later follow-up of this cohort (original number and age range were slightly different), 103 women developed CIN III. The OR for high-risk HPV (at baseline of the study) was 29 and was very high for persistent high-risk HPV infection (327) (Nobbenhuis *et al.* 1999). The Brazilian study was a retrospective analysis of 514 biopsies from cervical lesions of women who had been diagnosed with altered cytology (Cavalcanti *et al.* 2000) and who were followed for 8 years. Of 280 untreated patients, 82 women had progressed to carcinoma *in situ* or invasive cancer, 76 had lesions that regressed, and 122 had persistent lesions. HPV DNA was detected by *in situ* hybridization, which is not as sensitive as PCR-based methods. Risk estimates were reported for some HPV viral types; HPV-16 and 18 (combined) but not the combined HPV-31, 33, and 35 group were associated with CIN progression, and HPV-6/11 was inversely related to CIN progression.

3.3.2 Other anogenital cancers

Current studies on anogenital cancer other than cervical are presented in Table A-4 (Appendix A); they are organized by cancer site, followed by study design. All of these studies except for the one on anal cancer assessed HPV seropositivity, and thus most studies only assessed cancer effects to a limited number of HPV viruses, HPV-16 (all studies), HPV-18 (three studies), and HPV-33 (only one study).

3.3.2.1 Studies evaluating anogenital cancer at multiple sites

Epidemiological studies from different geographical locations and with different designs have reported a positive association between anogenital cancers and HPV seropositivity. Bjørge *et al.* (1997b) conducted a nested case-control study within a population consisting of two blood bank cohorts in Finland and Norway. HPV-16, 18, and 33 capsid antibodies were measured in serum donated at enrollment. Incident cases of vulvar/vaginal cancer, preinvasive vulvar/vaginal cancer, penile/scrotal cancer, and anal cancer were identified from cancer registries. Elevated risks were observed for HPV-16, 18, and 33 seropositivity (analyzed separately) as well as for development of all anogenital cancers (ORs for HPV-16 = 3.1, 95% CI = 1.4 to 6.9; HPV-18 = 1.2, 95% CI = 0.4 to 3.0; and HPV-33 = 2.8, 95% CI = 1.0 to 8.3) and for each specific cancer site. Higher risks were observed for HPV-16 seropositivity than for HPV-18 and 33 seropositivity at most cancer sites. Risk estimates for HPV-16 seropositivity were significant for all cancer sites except for the anus and penis/scrotum, although risk estimates for the latter were high (~7). ORs for HPV-18 seropositivity were only significant for preinvasive vulvar/vaginal cancer (OR = 12), and no ORs were significant for HPV-33 seropositivity. Highest risks for HPV seropositivity (regardless of type) were observed for preinvasive vulvar/vaginal tumors.

Carter *et al.* (2001) calculated risk estimates for HPV-16 and 18 seropositivity in six seroepidemiological case-control studies of anogenital cancers (cervical, vulvar, vaginal, and anal in females, anal and penile in males) conducted in the United States (cervical cancer was discussed in Section 3.3.1). Cases numbered from 88 (anal cancer in female) to 535 (vulvar cancer). HPV-16 seropositivity was significantly associated with an increased risk of cancers at all sites, and HPV-18 seropositivity was significantly associated with cancers at all sites in females (vaginal, vulvar, and anal). Strickler *et al.* (1998) reported that HPV-16 seroprevalence was higher in cases of cancers of the penis (63%), vagina (27%), and vulva (27%) than in controls (4%) in a cross-sectional hospital-based study from the United States.

3.3.2.2 Studies evaluating cancers of the vagina and vulva

Hildesheim *et al.* (1997a) conducted a population-based, case-control study consisting of 23 cases of *in situ* and invasive vaginal cancer and 28 controls from the United States. Consistent with the study reported by Carter *et al.* (2001), HPV-16 seropositivity was associated with an increased risk of disease (OR = 3.5, 95 CI = 1.0 to 13). The risk was higher in individuals with higher antibody titers (OR = 33); however, estimates were based on a small number of cases.

Case-control studies of vulvar cancer (preinvasive or invasive) conducted in New York and Chicago (Sun *et al.* 1996, Hildesheim *et al.* 1997b) (both reports from the same study population) and Washington State (Madeleine *et al.* 1997) reported positive associations with HPV-16 seropositivity, consistent with the findings from the prospective study reported by Bjørge *et al.* (1997b) and the case-control study by Carter (2001). The strength of the association was dependent on the histological grade and stage. Higher risks were observed for VIN III (13.4) than for invasive cancer (2.9) (Hildesheim *et al.*

1997b). Weak, nonsignificant risks were observed for individuals with keratinizing squamous carcinoma of the vulva, which is usually seen in older women and is not related to sexual history, whereas stronger associations were observed with either basaloid or squamous cell carcinomas (Hildesheim *et al.* 1997b, Sun *et al.* 1996). A strong association was observed in individuals with high antibody titers (OR = 20) (Hildesheim *et al.* 1997b). HPV-18 and HPV-6 seropositivity were not associated with either invasive cancer or carcinoma *in situ* in the study from Washington State (Madeleine *et al.* 1997), in contrast to the positive associations observed for HPV-18 seropositivity in the studies by Bjørge *et al.* (1997b) and Carter *et al.* (2001).

3.3.2.3 Studies evaluating cancers of the penis and anus

In contrast to the studies reported by Bjørge *et al.* (1997b) and Carter *et al.* (2001), Wideroff *et al.* (1996) did not find HPV-16 seropositivity to be related to penile cancer risk in a study conducted in China. This study did not categorize individuals as being seropositive but instead compared the median antibody titers (as measured by ELISA absorbance), which were similar in both groups, between 55 cases and 60 controls.

Frisch *et al.* (1997) reported a strong prevalence of high-risk HPV DNA (89% in women and 65% in men) but not low-risk HPV DNA (4% in women and 6% in men) in 388 cases of anal cancer in a study in Denmark and Sweden. HPV DNA was not detected in 20 cancer controls (not anal cancer). Risk estimates could not be calculated because of the inadequate number of controls. This study is in agreement with the positive association between anal cancer and HPV-16 reported by Carter *et al.* (2001) described above.

3.3.3 Head- and neck-related cancer

Most of the analytical epidemiological studies on tumor sites other than anogenital cancer reported since the 1995 IARC report concern head- and neck-related cancer. For most other cancer sites, only case-series studies that measure HPV DNA in tumor tissue are available. Because they contribute little to the evaluation of causality, they are not discussed in this section. Analytical studies on head- and neck-related cancers are described in Table A-5 (Appendix A) where they are organized by tumor site(s). Some studies evaluated head and neck or oral cancers as a group, others are specific for a tumor site. Most studies evaluated only a limited number of HPV viruses, and some studies reported estimates for combined HPV viruses; HPV-16 was evaluated the most extensively.

3.3.3.1 Studies evaluating cancers of the head and neck and the oral cavity

Studies using different epidemiological designs, assessing HPV infection by different methods, and studying populations from different geographical locations have provided some evidence for an etiologic role of high-risk HPV in head and neck cancer. Gillison *et al.* (2000) conducted a case-case study of 253 HPV-positive and negative cases of head and neck SCC in Maryland (United States). HPV prevalence was 25% (62/253), with HPV-16 accounting for the majority of HPV positivity. Oropharyngeal, compared to nonoropharyngeal, cancers and poor tumor grade were associated with HPV DNA

positivity in multivariate analyses. Ninety-four percent of the HPV-positive oropharyngeal tumors arose from the palatine or lingual tonsils. Oropharyngeal, HPV-positive tumors were negatively associated with smoking, alcohol use, and p53 mutations, but were positively associated with basaloid histopathology, and had an improved disease-specific prognosis, suggesting that they constitute a different molecular, clinical, and pathological disease pattern than HPV-negative oropharynx tumors. Alcohol use and smoking are risk factors for head and neck cancer.

High-risk HPV infection (mainly HPV-16), as measured by DNA, was associated with an increased risk of head and neck cancer (OR = 4.3, 95% CI = 1.3 to 14.8) in a hospital-based, case-control study in Japan, although the number of cases was relatively small (n = 74) (Nishioka *et al.* 1999). Supporting these studies and providing temporal evidence for a role in HPV infection and head and neck cancer, Mork *et al.* (2001) reported elevated risks for HPV-16 seropositivity but not for HPV-18, 33, or 73 seropositivity in a prospective (nested case-control) study from Norway, Finland, and Sweden. This study had a sufficient number (n = 292) of cases to stratify by tumor site; significant elevated risks were observed for tumors of the tongue, oropharynx, and larynx. However, a prospective seroepidemiological study from Finland (Dillner *et al.* 1995) did not report a difference in HPV-16 seroprevalence between head and neck cases (as a class) and controls, although the development of a specific tumor (esophageal, see below) was strongly associated with HPV-16 seropositivity.

Two population-based studies from the United States with a reasonable number of cases from Washington state (Schwartz *et al.* 1998) and Iowa (Smith *et al.* 1998), measured HPV DNA in oral cancer patients and control subjects. HPV DNA detection was significantly associated with that cancer in the Iowa study (OR = 3.7, 95% CI = 1.5 to 9.3) but not the one from Washington (OR for high-risk HPV = 1.3, 95% CI = 0.6 to 2.9). However, HPV-16 seropositivity was associated with oral cancer risk (OR = 2.3, 95% CI = 1.6 to 3.3) in the latter study. The lack of association with HPV DNA detection may result from factors relating to sampling and the nature of the biomarker. HPV DNA detection may be a poor measure of past exposure. The use of exfoliated cells in cases may have underestimated the prevalence because cells harboring HPV may have been removed by tumor biopsy due to the focal nature of HPV infections (Schwartz *et al.* 1998). Similar to the result observed for some anogenital cancer, a synergistic interaction was observed between HPV (antibodies) and smoking (Schwartz *et al.* 1998) in this study.

3.3.3.2 Studies evaluating specific head and neck tumors

Specific head and neck tumors that have been evaluated include the tonsils and esophagus. Mellin *et al.* (2000) reported a higher prevalence of high-risk HPV DNA in 60 patients (43%) with tonsillar cancer than in 10 noncancer patients (0%) in a Swedish study. This study was hospital based and assessed HPV exposure by detection of HPV DNA.

Two case-control studies, a population-based study in Sweden (Lagergren *et al.* 1999) and a hospital-based study in China (Han *et al.* 1996), and two prospective, nested case-

control studies in Norway (Bjørge *et al.* 1997a) and Finland (Dillner *et al.* 1995) investigated HPV serology in esophageal cancer. HPV-16 serology was positively associated with risk in the hospital-based study (significantly) and the two prospective studies (significant in the Finnish study, not significant in the Norwegian study) but not in the population case-control study from Sweden. The Finnish study reported a much higher risk estimate (OR = 13.1) than the other positive studies (ORs < 5), but this may be the result of a lower prevalence of HPV seropositivity in controls (2.5 compared to 7 to 11 in the other studies) rather than a higher prevalence in cases (21 compared to 21 to 24 in the other positive studies). Risk estimates increased in individuals with higher antibody titers, as assessed by using a higher absorbance cutoff for defining positivity, in the Chinese and Norwegian studies. HPV-18 and 33 seropositivity also were associated with an increased risk of esophageal cancer, the latter being significant in the Norwegian prospective study. In contrast, a decrease in risk was observed between HPV-18 seropositivity and esophageal cancer in the Swedish population-based study. Advantages of the Swedish study compared to the European prospective studies are a larger number of cases (173) and the use of multivariate analyses. The Finnish study reported a very strong risk estimate, but this needs to be interpreted with caution due to the small number of cases (39) and a lower HPV seroprevalence in controls than in other studies. Strengths of the Norwegian study are its prospective design and the observation of an exposure-response relationship with respect to HPV-16 antibody levels. The two prospective studies adjusted for smoking, and the Chinese study only adjusted for age.

3.4 Viral characteristics: viral persistence, multiple infections and variants

Studies on viral persistence and variants are described in detail in Appendix A, Tables A-6 and A-7.

3.4.1 Viral persistence

Women who do not clear HPVs infection within 1 to 2 years are at risk for long-term persistent infections (Einstein and Burk 2001). Follow-up studies, of both asymptomatic and symptomatic women who donated more than one cervical smear at different time points, demonstrated that high-risk HPV DNA persistence is critical to SIL persistence (Ho *et al.* 1995) and the development of high-grade SIL, carcinoma *in situ*, or cervical cancer (Moscicki *et al.* 1998, Schlecht *et al.* 2001, Wallin *et al.* 1999, Ylitalo *et al.* 2000a). Low-risk HPV infections are less likely be persistent (Moscicki *et al.* 1998). In a follow-up study of women from the United States, the majority of persistent viral infections (95%) that led to SIL persistence were caused by high-risk viruses (Ho *et al.* 1995). Type-specific persistence, defined as positive for the same viral strain in two smears, was associated with a greater than 50-fold increased risk of developing invasive cervical cancer (Wallin *et al.* 1999). In this study, the second smear was taken after cancer development. Risk estimates are lower in viral persistence studies, in which consecutive smears are at the beginning of the study; risk estimates for SIL (any SIL) increased from approximately 10, for viral persistence defined as positive for first two visits after enrollment, to 95, for viral persistence defined as positive at both enrollment and diagnosis.

Viral persistence is more likely to be caused by high-risk HPV infection, specific HPV variants, and high viral load, and to occur more often in older women (Villa 1997). Women with low-risk HPV-type infection were more likely to show regression than those with infection caused by high-risk HPV (Moscicki *et al.* 1998). Nevertheless, defining persistence is often difficult because the possibility that the same HPV virus detected at a later follow-up is due to a new infection of the same HPV type cannot be ruled out. (Villa 1997).

3.4.2 Multiple infections

Case-control studies reviewed in Section 3.3.1.2 varied in their estimates of multiple infections, with multiple infections occurring in 1% to 23% of the controls and 7.9% to 32% of the cases. Some studies have reported higher risks for multiple infections (Chan *et al.* 1999, Herrero *et al.* 2000, Santos *et al.* 2001), whereas other studies have reported higher risk for a single infection (Chaouki *et al.* 1998, van der Graaf *et al.* 2002). Multiple infections may make it difficult to ascertain risks due to individual HPV types; for example, Nglelangel *et al.* (1998) reported a positive association with HPV-66 and cervical cancer, but HPV-66 was only detected as a coinfection with other high-risk types. Nevertheless, given the high odds ratios, the somewhat low prevalence of multiple types, and the fact that most authors have reported when a type was not detected as a single infection, it is unlikely that multiple infections have very much impact on determining risk for individual HPVs.

3.4.3 Variants

As summarized in Table 3.2, studies of different epidemiological design (prospective or case-control) and in different locations (Korea, England, Costa Rica, United States, or Brazil) reported that sequence variants in the HPV-16 LCR (Hildesheim *et al.* 2001b, Villa *et al.* 2000), E5 (Bible *et al.* 2000), E7 (Song *et al.* 1997), or uncharacterized mutations (detected by single-strand conformation polymorphism [SSCP]) (Xi *et al.* 1997) modified high-grade CIN or cervical cancer risk compared to the prototype sequence. HPV-16 E6 sequence variants also have been associated with CIN progression (Matsumoto *et al.* 2000).

HPV-16 variants have been characterized from samples collected from various geographical locations and classified phylogenetically according to the location; e.g., European prototype (also found in North America), African 1 and African 2 variants, Asian variants, and Asian-American variants (which occur in Central and South America and parts of Europe such as Spain) (Villa *et al.* 2000). Non-European sequence variants in HPV-16 LCR (occurring between nucleotides 7495 and 7886) were associated with an 11-fold increased risk of cervical cancer compared to the European prototype sequence in a Costa Rican study (Hildesheim *et al.* 2001b). Similarly, non-European sequence variants (OR = 22.1) were associated with a higher risk than European prototypes (OR = 6.1) for developing high-grade CIN in a prospective study from Brazil (Villa *et al.* 2000). Non-European variants in this study also had a higher risk of persistence (OR = 5.4) than the European prototype (OR = 2.2).

Table 3-2. HPV variants

Region of Variant	Variant(s)	Effect	Geographical location	Reference
HPV-16	Uncharacterized	Positive association with CIN II/III	United States	Xi *et al.* 1997
HPV-16 and HPV-18	European Non-European	High-grade SIL, RR = 6.1 Persistent HPV infection, RR = 2.5 High-grade SIL, RR = 22.5 Persistent HPV infection, RR = 4.5 Reference group included HPV-16-negative women	Brazil	Villa *et al.* 2000
HPV-16 LCR	Non-European	OR = 11 (cervical cancer) Comparison group HPV-16-positive women with European variants	Costa Rica	Hildesheim *et al.* 2001b
HPV-16 E5	Variant 5	Negative association with CIN Decreased RNA expression (not significant)	England	Bible *et al.* 2000
HPV-16 E6	T/G Leu to Val	No association with persistent HPV-16 infection or CIN progression No association with cancer risk Positive association with CIN severity Negative association with CIN severity	The Netherlands England and Scotland Sweden Italy	Bontkes *et al.* 1998 Cuzick *et al.* 2000 Zehbe *et al.* 2001 Zehbe *et al.* 2001
HPV-16 E6	D24E L83V	CIN progression, OR = 4.8 CIN progression, OR = 3.0 (CIN I compared to CIN III)	Japan	Matsumoto *et al.* 2000
HPV-16 E7	KE7	More common in HPV-16-positive cancer than noncancer lesions	Korea	Song *et al.* 1997

3.5 Cofactors

Persistent high-risk HPV infection appears to be critical for cervical cancer development. The strong ORs observed for HPV infection are unlikely to be due to confounding by host factors. Nevertheless, not all individuals infected with high-risk HPV viruses develop cervical cancer, suggesting the importance of genetic factors and environmental or lifestyle factors that promote HPV persistence and carcinogenicity. Moscicki *et al.* (1998), in a study of the natural history of HPV infection, reported that 90% of the women with persistent HPV did not develop high-grade SIL. Studies on cofactors, including HLA variants, and studies in HIV populations are described in detail in Appendix A, Tables A-8 (HLA variants), A-9 (cofactors), and A-10 (HIV populations).

3.5.1 HLA variants

In this section the review is limited to studies reporting risk estimates for HLA variants in HPV-positive cases. Table 3.3 summarizes the findings for Class II alleles and haplotypes.

HLA antigens are involved in presenting antigen peptides to T lymphocytes and thus may modulate viral disease susceptibility (Oldstone 1991, cited in Ferrera *et al.* 1999b). HLA class I antigens (A, B, and C) are expressed on all nucleated cells, and HLA class II antigens (DR, DQ, and DP) are primarily expressed on antigen-presenting cells (Wang *et al.* 2001). Recent studies suggest that some HLA alleles may modulate HPV carcinogenesis; however, it is difficult to find a consensus among studies due to different patient populations, which vary with respect to ethnic makeup, stage of disease, assessment of HPV positivity (serology or DNA), and the complexity of number of different alleles and haplotypes.

Both HLA Class I and Class II antigens have been studied in HPV-positive CIN cases and/or cervical cancer. Class I alleles, B7 (Hildesheim *et al.* 1998, Wang *et al.* 2001) and B63 (Krul *et al.* 1999), were reported to increase the risk of HPV-associated cervical neoplasia, and Class B*44 was reported to increase the risk of CIN progression (Bontkes *et al.* 1998). Some HLA Class II alleles (see Table 3-3 for references on Class II alleles and haplotypes) such as DQA1*0102, DQB1*03, DRB1*0401, and DRB1*07 have been suggested to confer risk, whereas other Class II HLA alleles, such as DQB1*0603 and DRB1*13, have been suggested to confer protection to HPV-associated CIN and/or cervical cancer, with the most consistent findings probably for DQB1*03 and DRB1*13. Conflicting results have been reported for HLA DQB1*0602 and DRB1*15 alleles; some studies have reported a positive association, while others have reported a negative association with CIN and/or cancer risk. Individuals who have both the Class I B7 and Class II DRB1*0302 were had an 8-fold higher risk of high-grade SIL.

DRB1*1301 and DQB1*0603 and the DRB1*1501, and DQB1*0602 have been reported to be in linkage disequilibrium in some populations (Wang *et al.* 2001). HPV-positive women with both the DRB1*1301 and DQB1*0603 alleles had a decreased risk of HSIL or cancer (OR = 0.1, 95% CI = 0.01 to 0.6). Studies evaluating the DRB1*1501/DQ*0602 haplotype have reported conflicting results with respect to

whether this haplotype is protective or a risk factor. Other haplotyes also were reported to modify CIN and or cancer risk; DQA1*0102/DQB1*0602, DRB1*1101/DQB1*0301, and DRB1*0401/DQB1*0301 were associated with increased CIN risk, but the DQA1*0102/DQB1*0604 was associated with decreased CIN risk.

HLA alleles also have been studied with respect to HPV infection. The HLA Class II antigens, DRB1*07, DRB1*1501, and DRQ1*0602, have been reported to increase the risk of HPV infection (Bontkes *et al.* 1998, Beskow *et al.* 2001), and the DRB1*1501 and DRQ1*0602 alleles are associated with long-term HPV infection (Beskow *et al.* 2001).

The impact of HLA allele on cervical cancer risk may be more important for some HPV variants. For example, the presence of non-European variants was significantly associated with detection of the HLA DRB1*1102-DQB1*0301 haplotype in Costa Rican women (Herrero *et al.* 2000), and the HPV 350 T/G variants were associated with the HLA DRB1*07 allele (Bontkes *et al.* 1998) in European women and with the HLA DRG1 1501 allele (or haplotypes containing the allele) in Scottish and English women, although the latter association was not significant (Cuzick *et al.* 2000). While the specific HLA haplotypes participating in HPV-associated carcinogenicity are not well characterized and mechanisms have not yet been elucidated, it seems feasible that they may be one of the many immune genes involved in HPV infection and cervical neoplasia because they code for antigens involved in the immunological responses against HPV infections.

Table 3-3. Risk (RR, OR, and other) of Class II HLA alleles (including haplotypes) in HPV positive cases[a]

Study/ location	DQA1 *0102	DQB1 *03	DQB1 *0602	DQB1 *0603 & *0604	DRB1 *03 or *0401	DRB1 *07	DRB1 *13	DRB1 *15
Nested case-control studies								
Sanjeevi *et al.* 1996 Sweden	3.8 (1.4–10.8) Haplotype DQ6 (DQA1*0102/ DQB1*0602) 6.0 (2.0–18.1)		5.7 (1.9–17.1)					5.8 (1.9–17.6) Haplotype DR15/DQ6[b] 6.8 (2.0–23.4)
Hildesheim *et al.* 1998 USA		1.7 (0.8–3.5) (*0302)	8.8% cases vs. 23.4% in controls; *P* = 0.02				0.6 (0.3–1.3)	12.1% in cases vs. 23.9 in controls; *P* = 0.04 Haplotype DRB1*1501/ DQB1*0602 0.2 (0.1–0.60)
Beskow *et al.* 2001 Sweden			39% in cases vs. 27% in controls; *P* = 0.028[c]					40% in cases vs. 28% in Controls; *P* = 0.027[c]
Wang *et al.* 2001 Costa Rica		Combined effects of 0302 with B7 8.0 (1.6–40.7)		0.1 (0.03–0.6) *0603			0.2 (0.1–0.7) Combined effects with *0602 0.1 (0.01-0.6)	

Study/ location	DQA1 *0102	DQB1 *03	DQB1 *0602	DQB1 *0603 & *0604	DRB1 *03 or *0401	DRB1 *07	DRB1 *13	DRB1 *15
Case-control studies								
Helland *et al.* 1998 Norway	Haplotypes DQA1/DQB1 0102/0602 10.1 (1.3–449) 0102/0604 0.1 (0.0–0.9)		10.1 (1.3–44)	0.1 (0.0–0.9) *0604				
Brady *et al.* 1999 England						42% cases/vs. 24% in controls; *P* = 0.01		
Maciag and Villa 1999 Brazil					*03 1.9 (1.0–3.4)			2.3 (1.3–4.2)
Ferrera *et al.* 1999b Honduras			0.34, *P* = 0.04					
Cuzick et al. 2000 England and Scotland		2.5 (1.3–4.9) (*0301)			*0401 3.4 (1.6–7.3) Haplotype DRB1*0401/ DQB1*0301 4.8 (1.8-12.2)			1.7 (1.0–3.0) Haplotype DRB1*1501/ DQB1*0602 1.8 (1.0-3.3)

Study/ location	DQA1 *0102	DQB1 *03	DQB1 *0602	DQB1 *0603 & *0604	DRB1 *03 or *0401	DRB1 *07	DRB1 *13	DRB1 *15
Lin *et al.* 2001 Senegal		Haplotype DRB1*1101/ DQB1*0301 2.6 (1.0–7.0)					0.5 (0.2–1.1)	
Neuman *et al.* 2000 USA Family-based data		*P* = 0.006, transmission/ disequilibrium test						

[a]Information on haplotypes is described in column of one of the alleles contributing to the haplotype.

[b]DQ6 is DQA1*0102/DDQB1*0602 – see column DQA1*0102.

[c]Correction for multiple tests.

3.5.2 Environmental and lifestyle factors

Cofactors such as smoking, hormones, and immunosuppression may cooperate with HPV in the induction of cancer; these factors can modify HPV carcinogenicity at various stages, including susceptibility to HPV infection, HPV persistence, and CIN progression. Studies evaluating these factors vary with respect to design (prospective, case-control, progression), assessment of HPV (DNA and serological), analysis methods (multivariate and stratified analyses), disease endpoints (high grade CIN, cervical cancer, and other cancers), and geographical location. The recent case-control studies conducted by IARC in various geographical locations also assessed the role of possible cofactors, including hormones (parity and oral contraceptives), smoking, sexual behavior, and indicators related to socioeconomic status (family income, education, and Pap smear history). Lower socioeconomic status and not having had a Pap smear have been consistently found to be risk factors for developing cervical cancer in HPV-positive women (Chaouki *et al.* 1998, Ngelangel *et al.* 1998, Rolón *et al.* 2000, Thomas *et al.* 2001a, Thomas *et al.* 2001b).

Studies evaluating smoking have used different analysis methods; some studies looked at the combined effects of HPV and smoking, others calculated the ORs for smoking in HPV-positive women (stratified analyses), and still others calculated the ORs for smoking using multivariate analyses that control for HPV exposure. Because this review is only concerned with the effects of smoking in HPV-associated cancers, the first two methods of analysis are of primary interest. Studies evaluating the combined effects of smoking and HPV have reported higher risks for high-grade CIN (Kjellberg *et al.* 2000, Olsen *et al.* 1998), vulvar cancer (Madeleine *et al.* 1997), and oral cancer (Schwartz *et al.* 1998) in HPV-positive smokers compared to HPV-positive nonsmokers or HPV-negative smokers. An interaction was observed in three of these studies. The attributable portion of CIN in one study caused by the smoking-HPV interaction for CIN was estimated to be 74%, but this may not be relevant in populations where smoking is low (Olsen *et al.* 1998).

Smoking also is a risk factor in HPV-positive women for developing high-grade SIL, as shown in studies from Denmark (Kjær 1998) and Costa Rica (Hildesheim *et al.* 2001a) and for developing invasive SCC as shown in studies from Thailand (Chichareon *et al.* 1998) and Costa Rica (Hildesheim *et al.* 2001a). Conversely, studies from Morocco (Chaouki *et al.* 1998) and the Philippines (Ngelangel *et al.* 1998) did not report an association between smoking and invasive cancer. Smoking was associated with developing CIN III and cancer in a prospective cohort study of HPV-positive women in the United States (Castle *et al.* 2002). Smoking may interact with HPV through its mutagenic or immunosuppressant components, leading to viral persistence, genetic damage, and cancer development. Cigarette smoking concentrates have been reported to transform HPV-16-immortalized endocervical cells (Villa 1997).

Hormones (as assessed by parity and oral contraceptives) have been positively associated with HPV-related cervical cancer in some, but not all, studies. Oral contraceptives may be more important in the development of ACIS (Madeleine *et al.* 2001). Oral contraceptives were reported to be cofactors for HPV-associated invasive cervical cancer

in studies from Morocco (Chaouki *et al.* 1998), and parity was found to be a cofactor in case-control studies from Morocco (Chaouki *et al.* 1998), the Philippines (Ngelangel *et al.* 1998), and Costa Rica (Hildesheim *et al.* 2001a). A pooled analysis of HPV-positive women from eight case-control studies of invasive cancer and two studies of carcinoma *in situ* (IARC multicentric studies) found the odds ratio risk for using oral contraceptives to be 2.8 (95% CI = 1.6 to 5.4) for 5 to 9 years and 4.03 (95% CI = 2.1 to 8.02) for 10 years or longer (Moreno *et al.* 2002); the OR for seven full-term pregnancies was 3.8 (95% CI = 2.8 to 5.5); parity was only associated with SCC and not with ADC or ADS (Muñoz *et al.* 2002). Oral contraceptives and parity were not associated with developing CIN III and invasive cancer in a prospective study from the United States, albeit this was a low parity population (Castle *et al.* 2002). Hormones may modify HPV expression because the upstream regulatory region of HPV has some homology to hormonally induced regulatory elements (Villa 1997).

Finally, studies in HIV-populations support the role of the immune system in HPV-carcinogenesis. HIV-infected individuals are at increased risk for developing HPV-associated cancer, and a prospective follow-up study reported that one in five HIV-infected women developed SIL within 3 years (Ellerbrock *et al.* 2000, Frisch *et al.* 2000). Other studies demonstrated that immunosuppressed individuals, assessed by HIV status and T lymphocyte counts, were more likely to have persistent HPV infection (Ahdieh *et al.* 2000) and to develop HPV-related high-grade CIN than immunocompetent (HIV-negative) individuals (Cappiello *et al.* 1997, La Ruche *et al.* 1998). HIV appears to be more important as a risk for persistence of HPV-related SIL than for invasion. A follow-up study of women with low-grade SIL reported that women with both viruses were more likely to have persistent SIL than those with only one virus. HIV was a risk factor for persistence after controlling for HPV (La Ruche *et al.* 1999).

3.6 Evaluation of the evidence for causality

HPV DNA is found in virtually all cervical cancer biopsies (99.7%), suggesting that it may be a necessary cause for development of invasive cervical carcinoma (Walboomers *et al.* 1999). High-risk HPV strains, most belonging to the HPV-16 or HPV-18-related families, account for the majority of HPV strains detected in cervical cancer biopsies (Bosch *et al.* 1995, Walboomers *et al.* 1999).

3.6.1 Strength and consistency

3.6.1.1 HPV-16

3.6.1.1.1 Cervical cancer

The IARC monograph provided strong evidence for the causality of HPV-16 and cervical cancer. Recent case-control studies, similar to those reported by IARC, reported very strong ORs for detection of HPV-16 DNA and invasive cervical cancer; all of these studies reported positive associations with invasive cervical cancer with ORs ranging from 83 to 910. Moreover, these findings have consistently been reported in studies in different geographical locations (North America, South America, Africa, Europe, and Asia), study designs (e.g., prospective or case-control), and measures of HPV exposure.

Seroepidemiological studies support the findings of DNA studies, that is, a significant association between HPV-16 and high-grade CIN and/or cervical cancer, but the magnitude of the association is much lower. Positive associations were found in all prospective and case-control studies with ORs for invasive cancer usually close to 3. Thus, the evidence from serological studies is consistent. Most studies report the sensitivity of HPV serology to be approximately 50% to 75% (using HPV-DNA detection as a standard); thus, HPV serology may underestimate exposure (Carter *et al.* 2001). Moreover, individuals who resolve a low-grade infection may be seropositive. Persistent infection appears to be the critical factor for cervical cancer development (Chua *et al.* 1996) (see Section 3.4). ORs for HPV seropositivity in some studies are higher when analyses are restricted to HPV DNA-positive cases (Carter *et al.* 2001), which may be a better measure of persistent infections.

3.6.1.1.2 Other anogenital cancers

The literature on HPV and noncervical anogenital cancers has expanded since the IARC retrospective (1995). Most of the studies listed in Table A-2 (Appendix A) are serological studies, and most have concentrated on vulvar/vaginal carcinoma. HPV-16 seropositivity was consistently associated with vulvar carcinoma in all studies, including a cohort study and four case-control studies (Bjørge *et al.* 1997b, Carter *et al.* 2001, Hildesheim *et al.* 1997b, Madeleine *et al.* 1997, Sun *et al.* 1996) with risk estimates usually between 2 and 5. Stratifying by histological type showed that HPV-16 seropositivity was associated with basaloid and warty-type vulvar cancers but not with keratinizing SCC; the latter is found in older women and is not strongly related to sexual history or HPV infection (Hildesheim *et al.* 1997b, Sun *et al.* 1996). Similarly, HPV-16 seropositivity was associated with vaginal cancer in all studies reviewed, including a cohort study and two case-control studies that reported estimates of similar magnitude to that for vulvar cancer (Bjørge *et al.* 1997b, Carter *et al.* 2001, Hildesheim *et al.* 1997a). Higher risks were found for preinvasive vulvar/vaginal disease than for invasive cancer (Carter *et al.* 2001, Hildesheim *et al.* 1997b). Fewer studies were available that evaluated the role of HPV-16 in penile and anal cancer, and most reported positive associations, although not always statistically significant (Bjørge *et al.* 1997b, Frisch *et al.* 1997, Strickler *et al.* 1998, Wideroff *et al.* 1996). Overall, the literature supports the role of HPV-16 in anogenital cancer.

3.6.1.2 Other high-risk HPV types

3.6.1.2.1 Cervical cancer

The 1995 IARC evaluation of high-risk strains other than HPV-16 and 18 was limited by a paucity of studies reporting ORs specific for these strains; this was due to their lower prevalence. Recent case-control studies, most of which were initiated by IARC, have provided greater information on the relationship of individual high-risk strains other than HPV-16 and 18. Muñoz (2000) published the results of a pooled analysis, which calculated the ORs for individual HPV types and invasive cervical cancer. The analysis included the results from 11 of 13 IARC-conducted studies for which there were available data; five studies (two publications) were available for the 1995 IARC assessment, and five have been published since the IARC report (described in Section 3.3.1.2); one study has not been published, and HPV testing is still in progress in two

studies. Strong pooled ORs [95% CI not reported] were found for many individual high-risk strains and are as follows: HPV-16, OR = 150; HPV-18, OR = 182; HPV-31, OR = 60; HPV-33, OR = 78; HPV-35, OR = 35; HPV-45, OR = 151; HPV-51, OR = 43; HPV-52, OR = 146; HPV-58, OR = 79; and HPV-59, OR = 347.

Although a pooled OR was not reported for HPV-39 or 66, a strong risk estimate was reported for HPV-39 in the IARC-initiated study from Peru (OR = 177) (Santos *et al.* 2001), and significant ORs were reported for HPV-66 (OR = ∞) from the IARC-initiated study from the Philippines (Ngelangel *et al.* 1998). However, HPV-66 was only detected with co-infection with other high-risk HPVs types. The IARC-initiated study from Morocco reported an OR of 17 for the combined group of HPV 51, 52, 56, 58, and 59. Other studies on individual virus types (not part of the IARC-initiated studies) from various geographic locations support these findings; elevated ORs were observed for HPV-58 in a Chinese study (Chan *et al.* 1999) and HPV-16, 18, 31, 51, 52, and 58 in a Japanese study (Sasagawa *et al.* 2000).

Some case-control studies did not report ORs for individual HPV types but did report high ORs for pooled oncogenic (high-risk) types (Lorincz *et al.* 1992, Herrero *et al.* 2000), HPV-16 or 18-related virus families (Ferrera *et al.* 1999a), and the combined group of HPV-31, 33, 35, 39 (Thomas *et al.* 2001a). Thus, similar to HPV-16, strong and consistent associations have been observed for individual high-risk HPV viruses and for high-risk HPV viruses as a class. In addition to the viruses listed above in the pooled analyses (HPV-18, 31, 33, 35, 45, 51, 52, 58, and 59), studies evaluating high-risk viruses as a class usually include HPV-39, 56, and sometimes 68 and 66.

Most seroepidemiological studies have only evaluated a limited number of types, i.e., HPV-16, 18, and 33, and thus do not provide much information on the evaluation of individual HPV viruses or high-risk viruses as a class. As mentioned above, the magnitude of ORs in seroepidemiological studies is much smaller. In case-control and prospective studies, HPV-18 seropositivity was associated with a 2-fold increased risk of cervical cancer or CIN III (carcinoma *in situ*) and a 3-fold increased risk of ADC (including *in situ* cases); a weak association was found for HPV-33 seropositivity and invasive cancer (Carter *et al.* 2001, Kjellberg *et al.* 1999, Wang *et al.* 1997, Dillner *et al.* 1997).

3.6.1.2.2 Other anogenital cancers

As mentioned above, most of the studies on other anogenital cancer are serology studies, which provide little information for assessing individual HPV types other than HPV-16. Some evidence suggests that HPV-18 is associated with vulvar and vaginal cancer, with stronger risks observed for preinvasive (*in situ*) than invasive cancer, but the evidence is inconsistent across studies (Bjørge *et al.* 1997b, Carter *et al.* 2001, Madeleine *et al.* 1997). HPV-33 seropositivity was significantly associated with all anogenital cancer as a class (no longer significant after adjusting for HPV-16), and elevated, but not significant, ORs were observed for vulvar/vaginal and penile/scrotal cancers. Thus, while there is some evidence to support the role of HPV-18 and HPV-33 in other anogenital cancer, it is still limited.

3.6.1.3 Head and neck cancer

The etiological evidence for HPV-16 and other high-risk HPVs in head and neck cancers is weaker. Most of the studies reviewed in this section reported a positive association between HPV and head-neck cancers (either as a general class, a specific subset [oral], or specific tumor sites [oropharyngeal, laryngeal, and tonsillar cancer]). However, many of these studies are based on cross-sectional comparisons of DNA detection in a small number of hospital patients with and without cancer and thus provide limited or weak information for evaluating causality. Moreover, many studies used different samples for DNA detection in cases and controls, i.e., tumor biopsy from cases and exfoliated oral cells from controls (Dillner *et al.* 1997). Nevertheless, most of the available prospective seroepidemiological studies or population-based case-control studies confirm the finding of a role for HPV in head and neck tumors. Miller *et al.* (2001) conducted a meta-analysis of case reports and clinical series of oral SCC published from 1980 to 1998. For studies that reported prevalence of HPV in both normal tissue and precancer or cancer tissue, the OR for detection of HPV in oral SCC was 5.2 (95% CI = 2.5 to 10.9); the probability of detecting high-risk HPVs was 2.8 times greater than low-risk HPVs.

With respect to tumor sites, most studies, those evaluating a subset of head and neck tumors and those evaluating oral cancer as a subset, suggest that the strongest evidence for HPV may exist for oropharynx-related tumors. The evidence for esophageal tumors is less consistent; positive associations were found in three studies (a small hospital-based study (Nakagawa *et al.* 2000a) and two seroepidemiological prospective studies (Bjørge *et al.* 1997a, Dillner *et al.* 1995); no association was observed in a large, population-based case-control study (Lagergren *et al.* 1999). Sur *et al.* (1998) suggested that HPV may be more important in esophageal cancer in geographical areas associated with high risk, based on a greater prevalence of HPV from tumors in those areas; however, analytical studies have not been conducted to evaluate this hypothesis.

With respect to HPV type as evaluated in the more informative studies, seropositivity to HPV-16, but not to other high-risk types (18 and 33), was associated with the development of head and neck cancers (Mork *et al.* 2001). In contrast, elevated ORs for HPV-16, 18, and 33 seropositivity were associated with development of esophageal cancer in an independent study (Bjørge *et al.* 1997a). Most studies that measured HPV DNA reported high-risk HPV types to be the most common, and HPV-16 was the predominant type.

Exposure assessment for HPV (both DNA and serological) used in epidemiological studies is highly specific because the detection methods have both high sensitivity and high specificity (see Section 2). Most of the studies were well designed, used logistic regression analysis methods, and controlled for potential risk factors of cervical cancer. Therefore, the possibility of residual confounding due to unmeasured sources seems remote.

3.6.2 Temporality

It cold be argued that risk associations in case-control studies, which measured HPV in women after cancer diagnosis, result from the disease activating the virus or influencing the sampling and detection of the virus rather than from the virus causing the cancer. Cohort studies provide the most conclusive evidence for temporality by demonstrating that exposure (HPV infection) occurred before the development of cancer. The cohort studies consist of studies that followed women with normal cytology until the development of high-grade SIL (CIN II and III), precancer (CIN III or carcinoma *in situ*), or invasive cancer and longitudinal studies on CIN progression. As discussed in Section 3.1, most cases of untreated CIN III will progress to cancer; thus, this is an appropriate surrogate for evaluating invasive cancer. Studies that include cases of CIN II are probably less informative. Studies on invasive cancer are rare because most cohort studies (assessed by DNA) consist of women participating in screening programs; thus, women developing CIN would have been treated.

Most of these studies include small numbers of cases and therefore can only evaluate HPV-16 or high-risk viruses as a class. Significant positive associations between HPV-16 exposure (assessed by serology or DNA), HPV categories including HPV-16 (high-risk or HPV-18), and CIN or cancer development were consistently observed in all studies, albeit with considerable variation in the magnitude of the risk estimate. This variation in magnitude may come from imprecise risk estimates due to a small number of exposed cases, differences in cohort characteristics (HPV status, age), exposure assessment (DNA or serology), HPV grouping (16 only or high-risk), diagnosis (Pap smear or biopsy), and measurement of endpoint (high-grade CIN, CIN III, or cancer). Detection of HPV-16 DNA was associated with elevated risk for developing high-grade SIL or high-grade CIN in two longitudinal studies (one of which included HPV-16 and 18) (Schlecht *et al.* 2001, Woodman *et al.* 2001) and one nested case-control study (Liaw *et al.* 2001). The risk estimate in the nested case-control study was much higher (64) than in the longitudinal study that measured only HPV-16 (8.9). HPV-16 (assessed by DNA) also was significantly associated with the development of carcinoma *in situ* and invasive cancer, with ORs ranging from 15.8 to 104.8 (Wallin *et al.* 1999, Ylitalo *et al.* 1999, van der Graaf *et al.* 2002). One of the studies on invasive cancer was an archival study; and while the risk estimate was given for HPV, the majority of HPV detected was HPV-16 with only a few cases having 18, 31, 33, or 73 (Wallin *et al.* 1999); thus, the evidence is mainly for HPV-16. The highest odds ratio (104.8) was found for a study in the Netherlands of CIN and invasive cancer combined. HPV-16 seropositivity was associated with elevated risks for developing high-grade CIN, precancer, and invasive cancer in five seroepidemiological studies, with risk estimates ranging from 2.2 to 12.5 and with most estimates being close to three (Chua *et al.* 1996, Dillner *et al.* 1997, Lehtinen *et al.* 1996, Shah *et al.* 1997, Vonka *et al.* 1999). Lehtinen *et al.* (2001) conducted a meta-analysis of longitudinal studies evaluating HPV and cervical neoplasia. For seroepidemiological studies, the relative risk for HPV-16 and cervical cancer was 3.3 (95% CI = 1.1 to 4.9), and the population attributable risk was 27% for an unselected population and 44% for a low-prevalence population. Although there is a lack of consistency for magnitude of the ORs, the studies nonetheless provide strong evidence that HPV-16 infection precedes cancer development.

Risk estimates for other HPV types were reported in two DNA studies (high-grade CIN and carcinoma *in situ* [only 16 and 18]) and three seroepidemiological studies (18 and 33 only) (Chua *et al.* 1996, Dillner *et al.* 1997, Vonka *et al.* 1999, Woodman *et al.* 2001, Ylitalo *et al.* 1999). Elevated risks of high-grade SIL were observed for HPV-6 or 11, 16, 18, 31, 52, and 58 but not for HPV-33. There were a total of only 28 cases and thus probably a very small number of exposed cases for each HPV type, and as a result statistical significance was only observed for HPV-16, HPV-18, and the combined group HPV-6 or 11; the association with HPV-31 was of borderline significance. Half (14) of the 28 high-grade CIN cases had CIN II, which may be a less appropriate surrogate than CIN III for invasive cancer (Woodman *et al.* 2001). Detection of HPV-18 DNA was associated with a risk estimate of approximately two for developing carcinoma *in situ* (Ylitalo *et al.* 1999). HPV-18 seropositivity was associated with risk estimates ranging between two and three (borderline significance) for invasive cancer and CIN III, but little evidence of an association was found for high-grade CIN (Chua *et al.* 1996, Dillner *et al.* 1997, Vonka *et al.* 1999). A stronger association was found for ADC than for SCC in the study of invasive cancer (Dillner *et al.* 1997). A slightly elevated risk (1.5) also was observed for HPV-33 seropositivity and invasive cancer (Dillner *et al.* 1997). Evidence for temporality for other individual viruses is weaker than that for HPV-16, with the strongest evidence existing for HPV-18.

A larger number of DNA studies were available that evaluated high-risk HPVs as a class, but the endpoint of most of these studies was high-grade SIL. One study, with only 13 cases, reported a separate risk estimate for CIN III (Rozendaal *et al.* 2000). High-risk HPVs were consistently associated with high-grade SIL (or CIN), CIN III, and invasive cancer (Coker *et al.* 2001, Moscicki *et al.* 1998, Rozendaal *et al.* 2000, Schlecht *et al.* 2001, van der Graaf *et al.* 2002, Zielinski *et al.* 2001). Risk estimates in most studies for high-grade SIL were around 4, although one study reported a risk of 31. Risk estimates increased with measures of viral persistence (Moscicki *et al.* 1998, Schlecht *et al.* 2001). A very high risk estimate was observed in the CIN III case-control study (Rozendaal *et al.* 2000). Risk estimates for CIN III and invasive cancer (combined) were 28 (van der Graaf *et al.* 2002) and for invasive cancer (archival study) were 24 (Zielinski *et al.* 2001). In the meta-analysis of longitudinal studies (PCR) conducted by Lehtinen *et al.* (2001) (described above), the RR for oncogenic HPV infection and high-grade SIL/CIN II-III was 38 (95% CI = 14 to 100), and the risk for cervical cancer was 17 (95% CI = 8.2 to 33).

Studies on the progression of CIN to invasive cancer consistently reported strong positive associations with high-risk HPVs or the 16 and 18 combined group. The combined group of 31, 33, and 35 was not associated with SIL progression (Cavalcanti *et al.* 2000, Nobbenhuis *et al.* 1999, Remmink *et al.* 1995).

Case-control studies have reported much higher risk estimates for detection of HPV DNA than those observed in the cohort studies. Moreover, serological studies (both cohort and case-control) also report lower risk estimates, which are much more consistent between study designs. The discrepancies could be explained in part by viral persistence. As discussed in Section 3.4, longitudinal studies that measured multiple HPV at multiple time periods consistently reported higher risk estimates for viral persistence.

Furthermore, the archival cohort study on invasive cancer found a very high risk estimate of close to 60, which is more similar to the range observed in case-control studies, for type-specific persistence of HPV-16. Thus, the evidence suggests that it is persistent HPV infection that causes cervical cancer (Figure 3.1). As discussed in Section 2, DNA detection measures both recently acquired and persistent infections. For case-control studies, DNA positivity in cases is more likely the result of a persistent infection, whereas in cohort studies and in controls of case-control studies, DNA positivity is more likely to measure both newly acquired infections (most of which will be cleared) and persistent infections. Thus, risk estimates in cohort studies may be lower because only a subset of the exposure being measured (HPV infection) may be the actual risk exposure (persistent infection). Similarly, serological studies, both case-control and cohort studies, are measuring HPV exposure. This exposure may be the result of an HPV infection that resulted in low-grade SIL and regressed. Another possibility for the high risk estimates in the case-control studies may be differences in sample collection. For cases, HPV DNA was collected from tumor biopsy specimens, which have higher cellular yields than exfoliated cells collected from controls (Franco *et al.* 1999).

3.6.3 Coherence and biological plausibility

As discussed in Section 6, the carcinogenicity of HPV has been studied extensively. High-risk HPVs can immortalize and transform cells, cause transformation in transgenic animals, alter cell cycle progression by binding p53 and retinoblastoma (Rb) proteins, and alter growth regulatory pathways. Moreover, integrated HPV is found in human cervical tissue, and most invasive cancers contain HPV DNA; thus, there is strong evidence for biological plausibility.

3.7 Summary

Numerous case-control studies consistently demonstrate very strong associations with HPV-16, or high-risk HPVs as a class, and cervical cancer. Moreover, several recent case-control studies provided strong evidence of positive associations with individual high-risk HPVs (including HPV-18, 31, 33, 35, 39, 45, 51, 52, 58, and 59) and cervical cancer. There also is strong evidence of an association between HPV-16 and other anogenital cancers, especially vulvar cancer. Cohort studies have demonstrated that HPV-16 and high-risk HPV (as a class) infections occur before the development of high-grade CIN, CIN III, or invasive cancer; however, the evidence is weaker for high-risk HPVs as a class and for individual high-risk viruses due to a lower prevalence of these viruses. For the other HPV viruses, the evidence from cohort studies appears to be strongest for HPV-18. The strong ORs observed also preclude any likely confounding by other agents, and many studies have adjusted for most potential confounders. Lastly, the findings of epidemiological studies are supported by mechanistic studies that demonstrate that high-risk HPVs code for oncogenic proteins and can immortalize both cervical and epithelial cells (see Section 6).

Not all individuals positive for HPV infection develop cervical cancer. Most HPV infections are transient and are cleared within 1 to 2 years, and thus confer little risk for cancer development. The actual risk factor appears to be persistent HPV-16 or other high-

risk HPV infection. What causes HPV infections to persist probably depends on both viral characteristics, such as high-risk strains or variants, and cofactors. Recent studies are beginning to identify these factors and how they interact with HPV, but the exact mechanisms have not been elucidated. [Nevertheless, the evidence demonstrates a causal relationship between persistent HPV infection (high-risk) and cervical cancer.]

4 Studies of Cancer in Experimental Animals

IARC (1995) reviewed many studies that examined papillomaviruses isolated from monkeys, cattle, horses, deer, sheep, rabbits, rodents, dogs, cats, and birds and reported that several of the animal papillomaviruses were associated with malignant growth. Because of the species specificity of papillomaviruses, laboratory animals cannot be experimentally infected with HPV. However, transgenic animal models have been used to study HPV-dependent disease (Eckert *et al.* 2000). Transgenic mouse model systems have provided significant insights into the functions of the HPV E6 and E7 oncoproteins in cancer development and are discussed in Section 6. Section 4 focuses on the association between animal papillomaviruses and cancer. A review of the natural history and biological effects of these animal viruses is useful for understanding the carcinogenic potential of HPVs (IARC 1995). Selected studies of animal papillomaviruses are reviewed below.

4.1 Papillomaviruses in monkeys

Kloster *et al.* (1988) cloned and characterized two novel papillomaviruses from monkeys, one from a cutaneous papilloma in a Colobus monkey (CgPV-2), the other from a lymph node metastasis of a penile SCC in a rhesus monkey (RhPV-1). DNA analysis indicated that the RhPV-1 DNA was integrated into the cellular chromosomal DNA, whereas the CgPV-2 DNA was not. The Colobus monkey later developed SCC of the larynx; however, nucleotide sequences extracted from the tumor were closely related to RhPV-1 rather than to CgPV-2. These researchers noted that tumor type, tumor location, and the association of papillomavirus genomes in benign and malignant tumors were similar for monkeys and humans. In follow-up studies, Ostrow *et al.* (1990, 1995) demonstrated that RhPV-1 was sexually transmitted; they provided molecular and serological evidence for its presence in colonies of rhesus monkeys from several institutions. In a retrospective study of a colony of rhesus monkeys, evidence of RhPV-1 infection was found in 71% of the females who had sexual contact with the index male (Ostrow *et al.* 1990). Squamous cell carcinoma of the cervix and adenosquamous cell carcinoma of the endocervix were each found in one animal. Cervical biopsies from 10 animals (32%) revealed other lesions associated with papillomavirus-induced neoplasia. These included koilocytosis, loss of maturation, nuclear atypia, and increased mitotic activity. These researchers concluded that RhPV-1 is an excellent model for human oncogenic genital papillomaviruses because it shows a high degree of sequence homology with HPV-16, is sexually transmitted, and results in a similar spectrum of benign and malignant disease.

4.2 Papillomaviruses in cattle

Bovine papillomaviruses (BPVs) have been found throughout the world and induce papillomas of the skin, alimentary canal, eye, bladder, genitals, paragenital area, teats, and udder (IARC 1995). Two subgroups (A and B) and six types (BPV-1–6) have been described to date. Subgroup A viruses (BPV-1, 2, and 5) induce fibropapillomas; subgroup B viruses (BPV-3, 4, and 6) induce epithelial papillomas (Jarrett *et al.* 1984a, IARC 1995). However, the virus type was not specified in the earlier studies discussed

below because the heterogeneity of BPV was not known until the late 1970s (IARC 1995).

4.2.1 Subgroup A BPVs

The DNA of BPV-1, 2, and 5 were characterized using restriction enzymes and physical mapping. Cross hybridization experiments indicate that BPV-1 and 2 share about 50% of their genome, while BPV-2 and 5 share at most only 5% of their DNA sequences (Campo *et al.* 1981). Each viral type is strongly associated with a specific lesion. BPV-1 is associated with fibropapillomas of the paragenital and genital area, BPV-2 is associated with fibropapillomas of the neck and shoulder, and BPV-5 is associated with rice grain fibropapillomas of the teats and udder (Campo *et al.* 1980, Campo *et al.* 1981, IARC 1995). BPV-2 has been reported in the alimentary tract of cattle, but these tumors do not produce infectious viral progeny (Jarrett *et al.* 1984b).

BPV-1 was isolated from a fibropapilloma on the udder of a cow and subcutaneously injected as a suspension in phosphate-buffered saline into 2-month-old hamsters (Pfister *et al.* 1981). The purpose of that study was to investigate the state of viral DNA in non-virus-producing tumors caused by papillomavirus infection. Two of six animals developed tumors at the injection site at 14 months. The tumors were removed after 4 months, and part of one tumor was transplanted to a second group of young hamsters. After 3 months, biopsied tissue from one animal in the second group was transplanted into a third group of hamsters. Histological analysis was conducted on two tumors from the first group and one each from the second and third groups. Tumors in the first group were described as a fibrous histiocytoma with tendency to fibrosarcoma in some parts and a fibroma with an increased number of collagen fibers and partially atypic fibroblasts. Tumors in the second and third groups were true sarcomas. No virus particles were detected in extracts of ground tumor material; however, tumor DNA was analyzed by the Southern blot technique, treated with restriction enzymes, and purified with cesium chloride-ethidium bromide gradient centrifugation. These tests demonstrated that the majority of viral DNA persisted as complete virus genomes and that there was no evidence for integration into cellular DNA.

Spradbrow *et al.* (1987) studied 13 dairy cattle in Australia that had skin lesions ranging in severity from benign scaly and horn-like outgrowths to squamous-cell and basal-cell carcinomas. Lesions occurred only on unpigmented or lightly pigmented areas of the skin. Early lesions in two of these animals progressed to SCC over a 3-year period. Eight animals were eventually euthanized because of the severity of the lesions. The lesions were confined to the skin in six animals but had penetrated the dermis and spread to the underlying muscle in two others. Viral DNA was detected in 10/11 keratotic lesions and 5/8 carcinomas. Although the viral DNA hybridized to BPV-1 under low-stringency conditions, the specific BPV type could not be defined. The researchers concluded that both papillomavirus infection and exposure to ultraviolet light were responsible for the skin cancers.

In one study, 7 of 15 naturally occurring bladder tumors from cattle grazing in bracken fern-infested areas contained multiple copies of episomal BPV-2 DNA (Campo *et al.*

1992). Chronic enzootic hematuria is often associated with cancers of the alimentary tract and bladder in cattle grazing in fields infested with bracken fern. Bracken fern contains mutagens, carcinogens, and immunosuppressants. Campo *et al.* (1992) designed a controlled study to investigate the cocarcinogenic action between BPV and bracken fern. Because the study was primarily designed to study the synergism of BPV-4 and bracken fern in alimentary tract cancer (see Section 4.2.2), animals were injected with BPV-4 rather than BPV-2. Thirty-six calves were divided into seven treatment groups and a single control group. A diet of bracken fern resulted in immunosuppresion as evidenced by a dramatic decline in the number of polymorphonuclear leukocytes and a chronic drop in circulating lymphocytes. All animals treated with azathioprine experienced a substantial drop in peripheral blood leukocytes and, because of severe immunosuppresion and hematuria, were sacrificed after 1 year. Quercetin had no effect on the immune status. Benign bladder hemangiomas occurred in all animals treated with azathioprine. Malignant bladder tumors (transitional cell carcinomas, hemangiosarcomas, or hemangioendotheliomas) were induced in all 12 animals given bracken fern. These tumors were indistinguishable from naturally occurring tumors. The 13 tumors (3 from the azathioprine and BPV-4 group and 10 from the bracken fern and BPV-4 group) were analyzed for the presence of BPV DNA. BVP-2 DNA was detected in nine (69%) tumor biopsies. None of the animals was inoculated with BVP-2; therefore, these results suggest that latent BVP-2 can be activated in immunocompromised animals. Stocco dos Santos *et al.* (1998) demonstrated that BPV-2 was transmitted both vertically to offspring and horizontally to other cows through infected blood.

4.2.2 Subgroup B BPVs

BPV-3, 4, and 6 cause true epithelial papillomas (Jarrett *et al.* 1984a). Very little is known about the natural history and transmission of BPV-3 (IARC 1995). Campo *et al.* (1980) first described BPV-4 and its association with alimentary tract cancer in cattle, and Jarrett *et al.* (1984a) isolated BPV-6 from an epithelial papilloma of the udder.

Campo *et al.* (1980) noted that areas with a high incidence of bovine alimentary cancers had a higher incidence of papilloma infection, a higher number of papillomas per animal, and a higher number of specific sites of infection compared to cattle from surrounding areas where alimentary cancers were absent. Alimentary tract papillomas were frequently found in young cattle, but carcinomas typically did not occur in cattle less than 7 years old. BPV-4 was isolated from papillomas, and preliminary data indicated that BPV-4 DNA may have been present in the carcinomas. In a later study, Campo *et al.* (1994) experimentally reproduced this alimentary cancer syndrome.

The geographical distribution of alimentary cancer in cattle in Scotland overlaps the distribution of bracken fern. These cattle frequently have adenomas and adenocarcinomas of the lower intestine and carcinomas and hemangiosarcomas of the urinary bladder. The experimental design used by Campo *et al.* (1994) was described above (Campo *et al.* 1992). The studies were conducted concurrently using the same animals and treatments. A persistent alimentary papillomatosis developed within 6 weeks in all animals that were infected with BPV-4 and immunosuppressed by a diet of bracken fern or s.c. injections of azathioprine; however, progression to cancer occurred only in two animals infected with

BPV-4 and fed bracken fern. In the immunocompetent animals, the papillomas regressed after 1 year. BPV-4 DNA was detected in the papillomas but not in the carcinomas. The researchers concluded that immunosuppression was necessary for the papillomas to persist and spread and that mutagens in the bracken fern were probably responsible for neoplastic transformation. Once transformation occurred, viral expression apparently was not required to maintain the malignant state.

BPV-6 causes frond epithelial papillomas and only infects the teats and udder (Jarrett *et al.* 1984a, IARC 1995). Three Ayshire bull calves with no known exposures to BPV were reared out of contact with other cattle and were given multiple injections of purified BPV-6 in all four teats at 3 months of age. At 28 days no changes around the injection sites were noticed, but at 42 days, raised, rough plaques of 3 to 4 mm in diameter were evident. At 92 days, fully formed frond papillomas of 3 to 15 mm in length covered the injection sites. Although these tumors are not known to undergo malignant transformation, they are persistent (IARC 1995).

4.3 Papillomaviruses in deer

Papillomaviruses have been isolated from cutaneous papillomas in several species from the Cervidae family. These include reindeer, elk, and deer (Moreno-Lopez *et al.* 1987, Eriksson *et al.* 1994). Reindeer papillomavirus (RPV) was extracted and purified from a cutaneous fibropapilloma on a Swedish reindeer (Moreno-Lopez *et al.* 1987). Syrian hamsters 2 to 3 months old were injected (s.c.) with purified RPV; they developed tumors at the site of inoculation within 3 to 5 months. Control animals inoculated with normal reindeer skin tissue preparation did not develop tumors. No metastases were observed, but histological examination identified the tumors as fibrosarcomas. In addition, mouse C127 cells inoculated with RPV developed transformed foci within 2 to 4 weeks after infection. Cellular DNA extracted from the RPV-induced tumors or the transformed C127 mouse cells contained episomal and circular RPV DNA.

4.4 Papillomaviruses in sheep

Papillomas were found in 25/200 rumens from sheep (Norval *et al.* 1985). Histological analyses of 500 papillomas revealed that all but four were fibropapillomas. An early SCC was detected in one sample, and squamous papillomas along with fibropapillomas were observed in three cases. Neither virus particles nor viral DNA were detected; however, 6/10 papillomas reacted to antiserum that was prepared against the putative ovine rumen papilloma virus.

A papillomavirus was associated with SCCs of the perineum in sheep (Tilbrook *et al.* 1992). Biopsies were collected from the perineal region (58 samples) or axilla (4 samples) of 41 sheep from two farms in Western Australia and examined for evidence of papillomavirus infection. Tissue samples included samples from normal skin, small lesions, and SCCs. Methods included gross morphology, histology, immunohistochemistry, and DNA hybridization using HPV-11, 12, 16, and 18 DNA probes. Overall, papillomaviral-like DNA sequences were detected in about two-thirds of the biopsies examined, including one of three biopsies from normal skin. However, HPV-homologous sequences were detected in 7/7 (100%) clinical cancers compared to 11/22

(50%) small lesions and in 15/16 (94%) SCCs compared to 3/8 (38%) benign hyperkeratotic lesions.

An outbreak of cutaneous papillomatosis occurred in Merino sheep in Patagonia, Argentina in 1995. Lesions occurred on the muzzle and legs. In a retrospective study, Uzal *et al.* (2000) examined skin samples collected from five of these sheep and identified virus particles in the nuclei of the stratum granulosum cells. In addition, about 25% of the cell nuclei gave a positive reaction to an anti-bovine papillomavirus antibody.

4.5 Papillomaviruses in dogs

During a 7-year period (1976 to 1983), purebred beagles from a single commercial breeder in the Northeastern United States received prophylactic injections of live, unattenuated, canine oral papillomavirus (COPV) to protect against natural infection. At 10 to 14 weeks of age the dogs were inoculated with COPV in the right gluteal muscle. Some dogs were revaccinated at between 10 and 24 months of age. Twelve of 4,500 dogs inoculated with COPV developed a single proliferative cutaneous lesion at the injection site (Bregman *et al.* 1987). The latency period for the appearance of the first grossly recognizable lesion ranged from 11 to 34 months. Induced lesions included epidermal hyperplasia, epidermal cysts, squamous papilloma, basal cell epithelioma, and SCC. All lesions were confined to the treated area, and neoplastic lesions occurred in 11 of the 12 animals. Electron microscopy did not detect any virions in the neoplasms. However, structural antigens for COPV were detected in 5/12 samples by the peroxidase-antiperoxidase (PAP) technique. These results suggest that COPV can induce neoplastic lesions; however, the overall incidence was low, and other factors may have been required for tumor development.

Teifke *et al.* (1998) examined 19 cutaneous and mucocutaneous papillomas, 29 oral SCCs, and 25 nonoral SCCs of dogs for the presence of papillomavirus antigens, COPV DNA, and p53 expression. PCR and nonradioactive *in situ* hybridization (ISH) methods were used to detect COPV DNA. More than half (9/16) of the oral and cutaneous papillomas, but only one of the SCCs, tested positive for papilloma virus antigen. Stained nuclei were limited to koilocytes in the upper stratum granulosum and extending into the stratum corneum. Papillomavirus antigens were not detected in controls nor in three penile papillomas. Overexpression of p53 was detected in two cutaneous papillomas and 26% (14/54) of the SCCs [note, the paper incorrectly reported 35% for SCCs]. PCR analysis indicated that COPV DNA was present in 53% (10/19) of papillomas and in 5.6% (3/54) of SCCs. ISH results were similar to the PCR results. The observation that most SCCs do not contain COPV DNA does not necessarily mean that COPV does not contribute to carcinogenesis. This finding is consistent with the “hit and run” model of cell transformation described by Smith and Campo (1988). Mouse C127 cells transformed by BPV-4 did not require viral DNA for maintenance of the transformed phenotype. Nevertheless, the presence of COPV DNA in some of the carcinomas supports the hypothesis that papillomas may progress to a neoplastic state. The role of COPV in p53 overexpression in cancer cells was not clear. Section 6 provides further discussion on the interaction of papillomaviruses and p53.

4.6 Papillomaviruses in rabbits

Papillomaviruses in rabbits include cottontail rabbit papillomavirus (CRPV) and rabbit oral papillomavirus (ROPV) in domestic rabbits. All ROPV-induced papillomas spontaneously regress and are antigenically unrelated to CRPV. Harvey *et al.* (1998) reported the first successful experimental infection of genital mucosa with ROPV.

CRPV was the first papillomavirus to be identified and isolated and was the first to be associated with carcinogenesis (Rous and Beard 1934, Rous and Kidd 1938, Rous and Friedewald 1944). CRPV causes papillomas within 1 to 6 weeks in 95% to 100% of infected rabbits. In wild rabbits, the papillomas regress in about 6% of infected animals but progress to squamous cancer within 12 to 18 months in 23% of infected animals. The papillomas persist but remain benign in the majority of cases. However, in domestic rabbits experimentally infected with CRPV, 40% to 60% develop SCCs in 6 to 12 months (IARC 1995). CRPV in the presence of other carcinogens (tar or methylcholanthrene) demonstrated a marked synergism with rapid malignant progression (Rous and Kidd 1938, Rous and Friedewald 1944).

Virus particles are present in cottontail rabbit papillomas, but the amount varies by as much as 1000-fold among animals. Papillomas are not usually transmitted from domestic rabbit warts; however, small amounts of virus are produced. Carcinomas of either species do not contain infectious virus, but small amounts of viral antibodies have been detected (Breitburd *et al.* 1997).

Han *et al.* (2000) successfully prevented carcinoma development from CRPV-induced papillomas by vaccinating rabbits with DNA plasmids encoding CRPV E1, E2, E6, and E7 genes. Sixteen rabbits were infected with CRPV, and the resulting papillomas were allowed to grow for 3 months. Approximately 4 months after the initial infection, eight rabbits were immunized by intracutaneous injection of plasmid DNA that contained the CRPV genes, while the remaining eight rabbits received injections with the vector only. The papillomas progressed to carcinomas within 8 to 13 months in all eight vector-control rabbits; however, papilloma growth was suppressed in vaccinated rabbits, only two of which developed carcinoma after 12 to 15 months.

4.7 Animal models in vaccine development

HPVs and animal papillomavirus do not cross species boundaries to cause infections; however, the similarity in the structures of the viral genome and the functions of the proteins encoded by viral DNA in both HPVs and animal papillomaviruses make the latter good models for testing of potential vaccine target proteins (see Section 2.7 for a general discussion of vaccines). Human immune responses in vaccine studies have been reported to be similar to those seen in animal studies (Lowy and Howley 2001). The targets selected for vaccine development have focused on the L1 and L2 capsid proteins and the E6 and E7 proteins, although E1 and E2 also have been used (Cornelison 2000).

The capsid proteins L1 and L2 are commonly used for prophylactic vaccine development to induce neutralizing antibody responses to destroy viruses before they can enter a host cell. Papillomavirus virions inoculated systemically can elicit highly immunogenic

responses, and expression of high levels of L1, or coexpression of L1 and L2, results in self-assembly of these proteins into viral-like proteins (VLPs) that have similar antigenicity to the intact virion (Lowy and Schiller 1998). Some studies have used HPV-16 VLP vaccine in an animal host (mice); others have studied animal vaccines in their natural host. Studies in mice have shown that treatment with HPV-16 VLP causes the production of neutralizing IgA and IgG, which are protective, and also causes specific T-cell proliferation. Successful trials with preventive vaccines using L1 VLPs were reported for several animal papillomaviruses, including CRPV, BPV-4, and COPV, in their natural hosts when the injections were made with either alum or Freund's adjuvant or without adjuvant. These studies have demonstrated that the L1 VLP vaccines protected against persistent infection and carcinoma when challenged with high-dose virus (CRPV model). The studies have generally shown that immunization confers 95% to 100% protection against high-dose virus challenges and that the immunity can be passively transferred via serum or IgG (Breitburd *et al.* 1995, Christensen *et al.* 1996, Suzich *et al.* 1995, Kirnbauer *et al.* 1996).

The E6 and E7 proteins are the main targets used for therapeutic vaccines because of the expression of these proteins in cervical cancer cells. These models include the use of plasmid DNA and construction of chimeric genes and mutated genes. Examples of chimeric constructions include E7-L1/L2 VLP combinations or E7 combined with genes that help target the DNA to the appropriate cells (Ji *et al.* 1999, Da Silva *et al.* 2001). In one tumor challenge test, an E7 mutant (reduced transforming activity) vaccine provided protection to 100% of the mice (Shi *et al.* 1999). Bubeník *et al.* (2001) reviewed the results of numerous studies in which HPV-16 vaccines protected animals against tumor challenge or induced regression of tumors. These studies included a number of delivery methods, including proteins or peptides, viral vectors and DNA, genetically modified tumor cells, and dendritic cells. The authors concluded that animal models have made a substantial contribution to the development of therapeutic vaccines to be used in treatment of HPV-16-associated tumors.

Although preclinical and clinical studies with E6 and E7 vaccines have demonstrated protective and therapeutic immune responses, they have not been as successful when clinical outcomes are used as the endpoint (Da Silva *et al.* 2001). These trials, however, were conducted in patients with end-stage cervical cancer who may be refractory to any treatment. As further advances in vaccine development are made, it is likely that animal models will continue to play an important role in the development of both prophylactic and therapeutic HPV vaccines.

4.8 Summary

Because of the species specificity of papillomaviruses, laboratory animals cannot be experimentally infected with HPV. Many studies have investigated the carcinogenic action of various animal papillomaviruses in both their natural and heterologous hosts. The evidence linking CRPV and carcinogenesis and cocarcinogenesis is especially strong. Inoculation with BPV-1 has induced neoplasms in the genitals, bladder, and brain of cattle; sarcomalike tumors in the skin of horses; and metastatic tumors in the skin, ear, and brain of Syrian hamsters. Two of the BPVs (BPV-2 and 4) present strong evidence

for a causal link to bladder and alimentary tract cancer in cattle immunosuppressed by feeding on bracken fern. In addition, evidence exists for carcinogenic activity of other animal papillomaviruses, including RhPV, COPV, and sheep papillomavirus. Studies with rhesus monkeys showed sexual transmission of RhPV. Animal models offer promise as systems for testing the efficacy of HPV vaccines. For example, growth of papillomas in cottontail rabbits infected with CRPV and later vaccinated with DNA plasmid containing CRPV genes E1, E2, E6, and E7 was subsequently suppressed. Successful trials with preventive vaccines using L1 VLPs also have been reported for CRPV, BPV-4, and COPV in their natural hosts.

5 Genotoxicity

As a biological agent, HPV can have various effects upon infected cells; however, few studies have examined the direct effect of HPV on mutations and the genetic stability of HPV-infected cells. An IARC Working Group reviewed the literature published prior to 1995 and found some studies that pertained to chromosomal instability and chromosomal abnormalities, but found no genetic toxicology studies that utilized prokaryotic or eukaryotic mutation assays. The studies on chromosomal instability and abnormalities were discussed in the context of molecular mechanisms of carcinogenesis in the 1995 IARC Monograph. Genotoxicity studies published after the 1995 monograph are discussed in Section 5.2.

5.1 Studies reviewed by the IARC (1995)

High-risk HPV-16-induced chromosomal abnormalities *in vivo* were indicated by the presence of specialized, atypical mitotic figures including multipolar mitoses, mitoses with coarsely clumped chromatin, and two- and three-group metaphases (atypical mitotic figures showing chromosomal material on both sides of the equatorial chromosomes in the metaphase stage) (Crum *et al.* 1984). This finding was in contrast to a study by Winkler *et al.* (1984), who observed atypical mitotic figures in HPV-positive (unspecified genotypes) cervical lesions; but the frequency of atypical mitotic figures was similar in HPV-positive and HPV-negative lesions. Also, Claas *et al.* (1992) showed that the presence of three-group metaphase figures in the cells of cervical lesion biopsies was not correlated with infection by high-risk HPV-16 and 18.

Changes in chromosome stability were consistently observed in *in vitro* studies. Human keratinocytes transfected with DNA isolated from high-risk HPV-16 exhibited aneuploidy with near triploid or near tetraploid chromosome numbers (Dürst *et al.* 1987a, Hashida and Yasumoto 1991). Transfection with low-risk HPV DNA did not induce changes in ploidy. Smith *et al.* (1989) showed that transfection with high-risk HPV-16 and 18 was associated with changes in the copy number of specific chromosomes, including loss or reduction of chromosome 13 and increased copies of chromosome 1 or 3. Other genetic abnormalities associated with high-risk HPV-16 include homogeneously staining regions (HSRs), double minutes (Popescu and DiPaolo 1990), and prematurely condensed chromosomes (Dürst *et al.* 1987a). Normal human fibroblasts transformed with a retroviral vector containing HPV-16 genes E6, E7, or E6 and E7 exhibited gene amplification, genomic rearrangement, and tumor progression (White *et al.* 1994).

5.2 Genotoxicity studies published after 1995: Chromosomal abnormalities

Examination of 60 cervical biopsies revealed no tetrasomy in low-risk (types 6, 11, 40, 42, 43, and 44) HPV-infected lesions. Tetrasomy was associated with types defined by the authors as intermediate-risk (types 31, 33, 35, 39, 51, 52, 58, 59, 66, and 68) and high-risk (types 16, 18, 45, and 56) HPV infection and altered expression of p53 (Giannoudis and Herrington 2000). Evidence for the role of p53 in HPV oncogenesis is described in Section 6.

Duensing *et al.* (2001) and Skyldberg *et al.* (2001) detected abnormal centrosome numbers in HPV-16-positive cervical lesions. Expression of HPV-16 E7 and E6 oncoproteins is associated with abnormal centrosome synthesis and accumulation of nuclear abnormalities, respectively (Duensing *et al.* 2001). These studies show a correlation between the increasing genomic instability in high-risk HPV infection and progressive centrosome abnormalities and aneuploidy.

Hidalgo *et al.* (2000) showed that infection with high-risk HPV (e.g., types 16 and 18) was associated with chromosomal imbalances at specific chromosomal regions in 12 primary cervical carcinomas and 12 tumor-derived cell lines. These chromosomal regions have been described as HPV integration sites or fragile sites (see Section 6). Using uterine cervix tumors, Heselmeyer *et al.* (1996) observed a gain of chromosome 3q in a severe dysplasia/carcinoma *in situ*. Results showed this chromosome to be overrepresented in 90% of the carcinomas and to have undergone a high-level copy-number increase (amplification). The authors concluded that the gain of chromosome 3q that occurs in HPV-16-infected, aneuploid cells represents a crucial genetic aberration at the transition from severe dysplasia/carcinoma *in situ* to invasive cervical carcinoma.

Fifty-three cervical carcinomas were analyzed for the presence and type of HPV infection and loss of heterozygosity (LOH) for 3p, 3q, 4q, 5p and 5q. Deletion of 3p was dependent upon high-risk HPV infection. Deletion of 5p was independent of high-risk HPV (types 16 and 18) infection (Mitra 1999). There was no correlation between particular LOH events and the presence or type of HPV (type 16 or 18) (Sherwood *et al.* 2000).

5.3 Summary

The IARC Working Group reviewed the literature prior to 1995 and reported that high-risk HPV infection was associated with genetic instability, chromosomal aberrations, and aneuploidy. Publications subsequent to 1995 have confirmed the association of high-risk HPV infections with chromosomal abnormalities, including tetrasomy, abnormal centrosome numbers, and chromosomal imbalances at chromosomal regions.

6 Experimental Data Concerning the Mechanism(s) of HPV-Linked Carcinogenesis

IARC (1995) reviewed many studies on the mechanisms and role of HPV in carcinogenesis. High-risk HPVs, those HPV types associated with infection of genital mucosal sites and with low- and high-grade squamous intraepithelial lesions and invasive cancer (e.g., HPV-16, 18, 31, 33, 35, 39, 45, 51, and 52) (see Section 1), can induce both immortalization and transformation of human and rodent cells. Viral oncoproteins may interfere with cellular regulators such as tumor suppressor proteins, and integration of HPV viral DNA into the cellular genome results in the deregulation of oncogene expression and activation of proto-oncogenes. Changes in the cellular genome, viral integration, or alterations of the viral promoter may also be important for malignant progression of HPV-infected cells. Cofactors such as herpes virus, hormones, and other carcinogens may cooperate with HPV infections and affect viral gene amplification, transcriptional activation, and progression to malignancy, respectively.

This section summarizes the current knowledge of the mechanisms of HPV-associated carcinogenesis. Recent studies have defined the roles of HPV viral oncoproteins in immortalization, transformation, and progression. The integration of HPV DNA disrupts the circular viral genome, resulting in changes in viral and possibly cellular gene expression. Studies on transgenic mice, carrying the HPV genome or specific HPV genes, have enabled the determination of the roles that individual viral proteins play in the formation of HPV-associated tumors (benign and malignant). Various cofactors, including other viruses and a variety of chemicals with mutagenic, carcinogenic, or immunosuppressive potential, may cooperate with HPV in infected cells to influence the progression of HPV infection. Immunocompetence is an important factor in controlling HPV-associated carcinogenesis, as evidenced by the increased prevalence of HPV-induced diseases in immunosuppressed individuals.

6.1 High- and low-risk HPVs

The classification of HPVs in high- and low-risk categories has been central to the investigation of the possible link between HPV infection and development of epithelial cancers. Zur Hausen (1996) reviewed the role of papillomaviruses in human cancer and summarized the following distinctions between high- and low-risk viruses:

1) Originally the assignment of HPV types to high- or low-risk categories was based on the frequency of their association with cervical and anogenital cancers.

2) Later it was shown that high-risk viruses can immortalize human keratinocytes, while low-risk viruses cannot.

3) High-risk HPVs have the ability to bind p53 and pRB while low-risk HPVs do not, [or do so at reduced efficiency].

4) High-risk HPVs induce chromosomal aberrations.

6.2 Integration of HPV sequences

Viral DNA is maintained episomally during its normal life cycle. Integration of the viral DNA disrupts the viral genome, and deletion of parts of the viral genome sometimes occur (Lazo 1999). A frequent consequence of integration is inactivation of the viral E2 gene, which results in removal of inhibition of expression of the E6 and E7 genes.

Integration of viral DNA into the cellular genome occurs frequently in cervical cancer cell lines (Schwarz *et al.* 1985) and in most HPV-positive tumors; it frequently results in disruption of viral DNA at the E2 ORF (zur Hausen 1991). Integration of the viral DNA into the host cell genome is highly correlated with malignant progression; however, HPV may remain in the episomal state in some carcinomas. HPV-18 is always integrated in invasive cancers, and HPV-16 is integrated in a majority of the cases (Cullen *et al.* 1991). Theunis *et al.* (1999) examined the physical state of HPV-16 in three cases of *in situ* ungual SCC. Although HPV DNA was integrated in two of the three cases, the DNA was episomal in the remaining case.

6.2.1 Effects of HPV integration on viral and cellular gene expression

Dürst *et al.* (1987a) reported that transfection of cloned HPV-16 DNA into human epithelial cells resulted in integration of HPV DNA at a single chromosome site; they also demonstrated that transcription occurred through the entire early region, including the E2 gene. In contrast, analysis of cervical cancer lines showed that integration of HPV-16 or 18 DNA during malignant progression resulted in disruption or deletion of the E2 gene, which acts as a transcriptional repressor in high-risk HPVs (Schwarz *et al.* 1985, Jeon *et al.* 1995, Corden *et al.* 1999). Schwarz *et al.* (1985) showed that the SW756 cancer cell line contains most of the HPV-18 genome with disruption of the viral early region in the integrated copy. No transcripts could be detected from the E2, E4, and E5 regions. Inactivation of E2 resulted in derepression of the HPV-18 viral genes E6 and E7. The E6 and E7 proteins target cellular growth regulatory proteins; this allows replication of viral and cellular DNA and cell proliferation, and reintroduction of the E2 protein from HPV-16 or 18 suppressed cell proliferation (Dowhanick *et al.* 1995).

Wells *et al.* (2000) showed that the growth inhibitory effects of HPV E2 overexpression in HeLa cells were overcome by the HPV-16 E7 protein and adenovirus E1A protein, and to some extent by the HPV-16 E6 protein. Since the retinoblastoma tumor suppressor protein (pRb) interaction domains of the E7 and E1A proteins were required, the authors concluded that the pRb protein must play a role in E2 growth arrest. Experiments showed that E2-mediated growth arrest resulted in cellular senescence in HPV-positive cells with the cyclin/cdk inhibitor p21CIP as a potential downstream E2 effector. Expression of HPV-16 E2 in HPV-16-transformed SiHa cells resulted in a decreased growth rate and, in the absence of serum, in apoptotic death (Sanchez-Perez *et al.* 1997).

Integration of HPV-16 DNA also enhances the stability of the viral message through inactivation of a destabilizing element contained in the 3' region of the viral mRNA (Jeon and Lambert 1995). This increased stability of E6 and E7 mRNAs resulted in enhanced viral E6 and E7 transcription. A higher level of E7 protein was found in human cervical

epithelial cells that contain integrated viral HPV DNA than occurred in a matched cell line that contains cells with episomal HPV DNA.

6.2.2 Effects on proto-oncogene activation and expression

6.2.2.1 myc

In HeLa and C4-1 cells, chromosome 8 contained HPV-18 DNA integrated 5' to the c-myc gene (Dürst *et al.* 1987b). The HPV-positive HeLa and C4-1 cells exhibited higher levels of c-myc mRNA relative to other cervical carcinoma cell lines, suggesting HPV-induced cis-activation of the cellular oncogene. Increased levels of c-myc mRNA were also detected in human oral keratinocytes transfected with and immortalized by HPV-18 DNA (Shin *et al.* 1994). However, it is not clear whether activation of c-myc expression in this case is caused by a viral integration event or by expression of the HPV E6/E7 proteins.

6.2.2.2 Other oncogenes

Popescu and DiPaolo (1990) showed that HPV-16 integration into the cellular genome was associated with chromosomal alterations, including translocations, deletions, achromatic lesions, and partial duplications. Viral sequences were integrated on both normal and abnormal chromosomes. In another study (Dürst *et al.* 1987b), HPV-16 integrated in chromosome 13q14→13q32 in SiHa cells, while the HPV-18 integration site in SW756 cells was in chromosome 12 (but not near Ki-ras2 gene), and chromosome 8 near the c-myc gene was the site in HeLa and C4-I cell lines. In human cervical tumor cells, integration occurred on chromosomes 3 and 20 near the c-raf-1 and c-src-1 proto-oncogenes, respectively. The authors suggested that HPV-16 and 18 were involved in malignant transformation of cervical cells through integration-induced activation of cellular oncogenes. The validity of this concept remains to be established.

6.2.3 Fragile sites

In a recent review, Lazo (1999) suggested that integration most likely utilizes nonhomologous recombination, as there is no significant homology between cellular DNA and HPV-16 and 18 viral DNA. The preference for site of integration is in chromatin regions where cellular DNA might be more accessible. The author noted that several integration sites are in chromosomal regions identified as fragile sites. These include chr 3p14-21 (FHIT gene), chr 8q24 (myc) and chr19p13 (Jun-B).

Thorland *et al.* (2000) used RS-PCR (restriction site-PCR) to analyze the relationship between HPV integration in cervical cancer and the position of the common fragile sites (CFSs). In HPV-16-positive cervical tumor specimens from seven patients, three of the tumors showed HPV integration in or near an Alu element, while one tumor had HPV integrated into a sequence with high homology to the α satellite family of tandemly repeated DNA normally found in centromeric regions. The remaining three tumors had HPV-16 integrated in locations near CFSs, and two of the three occurred within a CFS at the molecular level. The authors concluded that HPV-16 integrations in cervical tumors occur frequently within CFSs.

6.3 Immortalization, transformation, and progression of cells by HPV

Recombinant DNAs from HPV-16, 18, 31, and 33, but not HPV-6, immortalized normal human foreskin keratinocytes after cotransfection with the neomycin resistance gene (Woodworth *et al.* 1989). The immortalized cell lines contained integrated and transcriptionally active viral genomes, and transcripts were detected for both E6 and E7. A second publication (Woodworth *et al.* 1990) based on these cell lines reported that most, although not all, late passage (> 180 population doublings) cells immortalized by HPV-16, 31, or 33 formed dysplastic epithelia when transplanted into athymic mice. In contrast, cells immortalized by HPV-18 formed dysplastic epithelia at both late and early passages (< 40 population doublings). The authors suggested that the immortalization of a subpopulation of cells within HPV-infected tissue could result in an increased risk of neoplastic progression.

Shin *et al.* (1996) investigated the role of genetic instability in the oncogenic transformation of HPV-16 and 18-immortalized human oral keratinocytes. Genetic stability was determined by measuring effects on cell cycle control including p53 expression, transcription of WAF1/CIP1 and gadd45 (genes involved in cell cycle arrest and enhanced DNA repair), and mutation frequency. This section summarizes the results for mutation frequency. The authors exposed normal human oral keratinocytes obtained from two donors (NHOK and NHOK-1), HPV-immortalized oral keratinocytes (HOK-16B, 18A, and 18C), and an oral cancer cell line expressing mutant p53 (SCC-4) to the genotoxic chemical *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The immortalized cells were prepared by transfecting NHOK with cloned HPV-16 and 18 DNA. Spontaneous and induced mutagenesis frequencies were measured using the shuttle vector pS189 with supF as the mutagenic target. Cells were transfected with pS189 plasmids and, 24 hours later, were exposed to MNNG for 2 hours. The cells were cultured in fresh medium for 24 hours, and the plasmids were recovered and transfected into *Escherichia coli* strain MBM7070. Bacteria with the wild type supF plasmids produce blue colonies, while bacteria with a mutation in supF produce white or light blue colonies. The mutagenesis frequency (%) = 100 X [(the number of white and light blue colonies)/total number of colonies]. The results are summarized in Table 6-1.

Table 6-1. Spontaneous and MNNG-induced mutation frequencies in normal, HPV-immortalized and oral cancer cells

Cell lines	MNNG concentration (µg/mL)	Incidence[a]	Mutant frequency (%)
NHOK	0	8/16,785	0.047
	0.5	14/14,750	0.095
	1.5	27/15,048	0.179
NHOK-1	0	13/16,147	0.081
	0.5	18/15,883	0.113
	1.5	23/13,644	0.168
HOK-16B	0	18/16,872	0.107*
	0.5	71/20,534	0.346*
	1.5	85/15,187	0.560*
HOK-18A	0	40/22,538	0.178*
	0.5	121/21,080	0.574*
	1.5	146/16,631	0.878*
HOK-18C	0	23/15,880	0.150*
	0.5	102/18,078	0.546*
	1.5	111/15,080	0.736*
SCC-4	0	112/18,003	0.622*
	0.5	134/18,930	0.708*
	1.5	140/15,098	0.927*

Source: Shin *et al.* 1996.

MNNG - *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

*Significantly higher than the corresponding treatment group in NHOK (χ^2 test). Significance level not given by authors.

[a]Number of mutants/number of total colonies.

The HOK and oral cancer cells had spontaneous mutation frequencies approximately 2-fold and 8- to 12-fold higher, respectively, than the NHOK mutation frequencies. MNNG exposure enhanced the mutation frequencies in all the cell lines, but the frequencies in the HPV-immortalized cells and cancer cells were about 3- to 6-fold higher than in the NHOK cells. Furthermore, most of the increase was due to an increase in point mutations, particularly the G:C to A:T transition. The authors concluded that the oncogenic transformation of HPV-immortalized cells might be partly associated with genetic instability resulting in the loss of cell cycle control and inefficient repair of DNA damage caused by genotoxic agents.

6.3.1 Chromosomal integration of foreign DNA

Kessis *et al.* (1996) investigated whether HPV-16 E6 and E7 could increase the incidence of integration of foreign DNA. They investigated the integration frequency of a reporter plasmid in cells expressing either high-risk (HPV-16) or low-risk (HPV-6 and 11) HPV-

derived E6 or E7 genes. Cells expressing high-risk, but not those expressing low-risk, E6 or E7 genes exhibited a significantly higher frequency of reporter DNA integration than the parental cells. High-risk HPV (types 16 and 18) genomes frequently integrate into the host genome upon carcinogenic progression, and integration is associated with a growth advantage of affected cells (Jeon *et al.* 1995). Therefore, these data suggest that the HPV E6 and E7 proteins may facilitate integration of the viral genome and, hence, the carcinogenic progression of high-risk HPV-positive lesions.

6.3.2 Transforming potential of HPV viral proteins E5, E6, and E7

The HPV viral proteins E5, E6, and E7 may each play a role in the HPV-mediated transformation of cells. Summarized below is the possible transforming function(s) for each of these proteins. Expression of E6 and E7 is retained in cervical cancers, whereas E5 expression is frequently lost due to viral integration.

6.3.2.1 HPV E5

HPV E5 is proposed to have several possible transforming functions, including a role in epidermal growth factor (EGF)-related signal transduction. HPV-16 E5 was mitogenic when transfected into mouse fibroblasts (Leechanachai *et al.* 1992, Pim *et al.* 1992). In human keratinocytes, EGF-R recycling was increased by transfection with HPV-16 E5; the activation of EGF-R required the C-terminal 5 amino acids of HPV-16 E5 (Rodriquez *et al.* 2000). Rodent fibroblast cells also were transformed by HPV-16 E5, and recycling of EGF-R was increased over that in control cells (Straight *et al.* 1993). Increased cell proliferation in soft agar was noted in 3T3-A31 or NIH3T3 cells transfected with HPV-16 E5 and subsequently treated with EGF (Leechanachai *et al.* 1992, Pim *et al.* 1992). E5 from HPV-11 and 16 also was reported to repress expression of cdk inhibitor p21WAF-1 (Tsao *et al.* 1996), although the mechanism of this repression is unclear. Others have shown that keratinocytes transformed with HPV E5 exhibited decreased gap junction communication (Oelze *et al.* 1995). The authors hypothesized that this function of HPV-16 E5 may play an important role in the disruption of cell-to-cell communication, a trait observed in transformed cells. In addition, the HPV-16 E5 protein may bind to the vacuolar H+-ATPase, a membrane-associated protein that has been linked to EGF-R cycling (Conrad *et al.* 1993). However, the binding of E5 to the ATPase had no effect on EGF-R activation. It is unclear what effect E5 has on vacuolar ATPase function or if the interaction plays any role in transformation.

6.3.2.2 HPV E6

The E6 proteins from high-risk HPVs interact with multiple cellular pathways that may contribute to cellular immortalization, transformation, and tumorigenesis. Baby mouse kidney epithelial cells were immortalized by HPV-16 E6 in cooperation with EJ-ras (Storey and Banks 1993). von Knebel Doeberitz *et al.* (1992) showed that expression of antisense E6-E7 RNA in HPV-18-positive C4-1 cervical carcinoma cells was associated with loss of neoplastic growth characteristics. In addition, treatment of HPV-positive cervical cancer cells with E6-binding peptide aptamers caused apoptosis (Butz *et al.* 2000). The data suggest that E6 contributes importantly to the induction and maintenance of the transformed state. These functions have been reviewed by Liu *et al.* (1999) and

Rapp and Chen (1998). A focus of research on interaction of E6 proteins with cellular proteins involved the role of E6 in inactivation of p53 tumor suppressor protein; however, E6 proteins also affect telomerase and proteins involved in regulating apoptosis, cytoskeletal structure, cell polarity, signal transduction, and differentiation (Thomas *et al.* 1999). The interactions of HPV E6 proteins with p53, telomerase, and apoptosis regulatory processes are summarized below.

6.3.2.2.1 Interaction with p53

High-risk HPV E6 proteins can interact with p53 (Werness 1989), and it has been suggested that E6 transforms cells by altering p53. The E6 oncoproteins encoded by high-risk HPV-16 and 18 are able to promote degradation of p53 by a ubiquitin-dependent process (Scheffner *et al.* 1990, Band *et al.* 1991). The authors proposed that the increased degradation of p53, under direction of the viral E6 oncoprotein, contributed to the transforming activity of E6. The HPV types that are classified as low risk encode E6 proteins that do not induce degradation of p53. Degradation of p53 by high-risk HPVs is mediated by a cellular protein, E6AP. Neither E6 nor E6AP can bind efficiently to p53, but E6 binds to E6AP, and the E6/E6AP complex binds to p53 (Huibregtse *et al.* 1991, 1993). The E6/E6AP complex acts as a p53-specific ubiquitin ligase, and p53 is targeted for proteolysis by the 26S proteasome (Scheffner *et al.* 1993).

In HPV-16 or 18-positive cervical cancer cells, degradation of p53 depended entirely on the action of E6, as the Mdm-2-dependent pathway was inactive (Hengstermann *et al.* 2001), leading the authors to conclude that the growth of HPV-positive cancer cells requires the E6 protein to target p53 for degradation.

Sedman *et al.* (1991) examined the alternative splicing of the E6 ORF of HPV-16. The alternatively spliced protein product of the E6 gene of HPV-16, designated E6*, was not required for transformation, as a splice-defective mutant was active in cell transformation assays. Elimination of the splice site, however, did cause a reduction in the amount of E7 protein produced, while E6 protein increased. Thus, the E6* protein may regulate expression of full-length E6 and E7 proteins rather than affecting cell transformation directly. Pim and Banks (1999) showed that HPV-18 E6*I, an E6 protein produced by alternative splice acceptor sites, exhibited antiproliferative activity that correlated with the ability of E6*I to induce apoptosis in a p53-dependent manner. When E6*I was mutated to eliminate binding to full-length HPV-18 E6, the inhibition of proliferation of a cervical tumor-derived cell line by E6*I also was obviated.

The presence of HPV-16 E6 correlated with the absence of p53 mutations in oral SCCs but not with overexpression of p53 (Penhallow *et al.* 1998). The authors proposed that since HPV E6 down-regulates p53 (Scheffner *et al.* 1990), progression to a malignant stage does not require a mutation in p53. This finding is consistent with earlier reports showing that HPV-16 and 18-positive lesions frequently retain wild type p53 and pRb expression (Scheffner *et al.* 1991, Wrede *et al.* 1991).

6.3.2.2.2 Effect on apoptosis

High-risk HPVs replicate in terminally differentiated epithelial cells that have withdrawn from the cell division cycle. As a result, the cellular DNA replication machinery must be

reactivated. To avert the normal cellular response to inappropriate DNA replication, the p53-mediated process of apoptosis must be blocked.

Cellular damage resulting from ultraviolet B (UVB) radiation often leads to apoptotic elimination of the damaged cells. Jackson and Storey (2000) exposed HT1080 cells to UVB and used terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL assay) to detect apoptosis. Wildtype (vector-expressing) cells experienced > 20% cell death, while cultures transfected with plasmids encoding E6 proteins from HPV-5, 10, or 18 had very few TUNEL-positive cells. E6 from HPV-77 reduced the percentage of apoptotic cells, but approximately 10% cell death still occurred. Although the mucosal HPV-18 E6 completely blocked expression of p53, the cells transfected with E6 from cutaneous HPV types (HPV-5, 10, or 77) demonstrated upregulation of p53. The authors then exposed a p53-null cell line (Saos2) to UVB and reported that E6 from all four HPV types could still inhibit cell death. The authors concluded that the antiapoptotic effect of HPV does not require degradation of p53. Jackson *et al.* (2000) extended these studies by examining the expression of Bak, a proapoptotic effector protein, after exposure of HT1080 cells to UVB. Bak was detectable by Western blot in cells transfected with vector only, but not in cells transfected with plasmids coding for E6 from HPV-5, 10, 18, or 77. They further showed that HPV E6 proteins (from HPV-5 or 18) promoted proteolytic degradation of Bak and thereby abrogated the accumulation of Bak. The authors suggested that elimination of Bak protein by E6 results in decreased apoptosis, which could then promote tumor formation.

6.3.2.2.3 Expression of telomerase

Telomerase extends the life span of cells in culture, and continued addition of telomeric sequences may be a mechanism of immortalization as one step in the progression to tumorigenesis (Rapp and Chen 1998). For this reasons, researchers examined a possible interaction between HPV E6 expression, activation of telomerase, and immortalization of cells.

High-risk HPV-16 E6 proteins can induce expression of hTERT, the protein subunit of the human telomerase enzyme (Klingelhutz *et al.* 1996). Activation is at the level of transcription, but the exact mechanism is unclear. Mutagenesis studies showed that p53 degradation and telomerase activation are independent (Kiyono *et al.* 1998).

Nair *et al.* (2000) used immunocytochemistry to evaluate the expression of HPV E6, p53, and Ki 67 in 72 cervical tissue samples ranging from normal, benign cervical tissue through low-grade intraepithelial lesions, high-grade intraepithelial lesions, and invasive cancers. Telomerase was expressed in 85% of the invasive carcinomas and 61% of the high-grade SIL but in only 10% of the low-grade SILs and 7% of the normal cervical tissue samples. Increased telomerase expression was noted in tissues infected with HPV-16 or 18 and in tissues expressing HPV E6. The authors suggested that an association may exist between high-risk HPV infection, activation of telomerase and other cellular proteins, and the increased proliferation of cells in cervical lesions.

6.3.2.2.4 Other cellular targets of E6

HPV E6 proteins interact with several other cellular proteins. These include E6BP (ERC55) (Androphy *et al.* 1987), a putative calcium-binding protein of unknown function, and E6-TP1, a protein with a high degree of sequence similarity to Rap GTPase activating proteins, which serve as negative regulators of Rap-mediated mitogenic signaling (Gao *et al.* 1999). E6 also interacts with several members of PDZ domain-containing proteins, including the human homolog of the Drosophila tumor suppressor, discs large (DLG) (Kiyono *et al.* 1997, Lee *et al.* 1997), scribble (Nakagawa and Huibregtse 2000), and MUPP1 (Lee *et al.* 2000). These proteins have important functions in regulating epithelial cell polarity. In addition, E6 interacts with IRF-3, a transcription factor that plays a pivotal role in the regulation of the transcriptional response to interferon (Ronco *et al.* 1998). Other putative cellular targets of HPV E6 proteins include paxillin, an integrator of integrin signaling (Tong and Howley 1997, Vande Pol *et al.* 1998); the gamma subunit of the AP-1 complex, which is involved in the formation of clathrin-coated pits (Tong *et al.* 1998); the serine/threonine kinase PKN (Gao *et al.* 2000); the replication protein hMCM-7 (Kukimoto *et al.* 1998); and the transcriptional coactivator p300/CBP (Zimmermann *et al.* 1999, Patel *et al.* 1999).

6.3.2.3 HPV E7

The multiple interactions of HPV E7 with cellular growth regulatory proteins that may contribute to cell transformation have been reviewed by Zwerschke and Jansen-Dürr (2000). In addition to the tumor suppressor pRb, E7 also binds to the pRb-related pocket proteins p107 and p130. Interactions with the cyclin-dependent kinase inhibitors p21WAF-1 and p27KIP1, the S4 ATPase subunit of the 26S proteasome, and the glycolytic control enzyme M2 pyruvate kinase (M2-PK) also have been described. These and other potential interactions of E7 with cellular regulatory proteins may be involved in the actions of E7 to 1) override cell cycle checkpoint controls, 2) modulate the expression of cellular proteins, 3) deregulate cellular carbohydrate metabolism, and 4) modulate apoptosis.

6.3.2.3.1 Immortalization and transformation

HPV-16 E7 can transform a number of established rodent fibroblast cell lines, including NIH3T3 and rat-1 cells. In addition, E7 also can cooperate with a ras oncogene to transform primary rat embryo kidney cells. High-risk [not defined by the authors] HPV-encoded E7 genes can cooperate with high-risk HPV E6 genes to immortalize primary human epithelial cells, the normal host cell types of these papillomaviruses. HPV E6/E7 immortalized cell lines are initially nontumorigenic, but upon prolonged passages, tumorigenic variants arise (reviewed by Jones and Münger 1996).

6.3.2.3.2 Interaction with the retinoblastoma tumor suppressor

The retinoblastoma tumor suppressor protein, pRb, acts as a critical regulator of the process of entry into the DNA synthesis (S)-phase of the cell division cycle. Since cellular replication enzymes that are exclusively expressed during S-phase are critical for viral DNA replication, and given that viral replication takes place in cells that have terminally withdrawn from cellular replication, abrogation of pRb function is critical for the viral life cycle (reviewed in Alani and Münger 1998). The E7 protein of both low-risk

(HPV-6) and high-risk HPVs (HPV-16) are similar structurally and functionally to both adenovirus E1A protein and the large tumor antigen (Tag) of simian virus 40; however, the high-risk (HPV-16) HPV E7 proteins have a higher affinity for pRb than the low-risk HPVs (HPV-6) (Heck *et al.* 1992). Heck *et al.* (1992) demonstrated that a single amino acid difference in the pRb binding region of the E7 proteins of low-risk (HPV-6) HPVs versus the high-risk (HPV-16) HPV E7 pRb binding domains could alter the binding affinity of the proteins. When various HPV E7 mutants were cloned into an eukaryotic expression vector to test their ability to transform (in cooperation with ras) primary BRK cells, pRb binding affinity was closely correlated with transformation potential. However, mutations in E7 that inhibited binding to pRb did not prevent HPV-16 DNA from immortalizing primary human keratinocytes, suggesting that E7-pRb binding is not essential for the immortalization of primary keratinocytes (Jewers *et al.* 1992).

E7 neutralized or abrogated the inhibition of the cell cycle and blockage at checkpoints, while high-risk HPV (HPV-16) E7 ensured transition from G1 to S-phase and increased cell proliferation, a hallmark of transformation. Interaction with E7 induced the degradation of the pRb tumor suppressor protein (Boyer *et al.* 1996, Jones and Münger 1997, Berezutskaya *et al.* 1997). Additional studies showed that the ability of E7 to induce pRb degradation is a high-risk (HPV-16) HPV E7-specific activity that is closely correlated with transformation (Jones *et al.* 1997a, Gonzalez *et al.* 2001).

6.3.2.3.3 Effect on transcription factor E2F

Release of the transcription factor E2F from its complex with pRb protein has been proposed as a mechanism in the growth deregulation and transformation that accompany transfection of cells with high-risk (HPV-18) HPV E7 protein. von Knebel Doeberitz *et al.* (1994) measured the levels of the transcription factor E2F and noted a 0% to 40% decrease in the amount of free E2F when HPV-18 E7 was expressed in SW 756 cervical carcinoma cells. They suggested that the partial E7-mediated release of E2F may be relevant for the transformation but that complete disruption of pRb-E2F binding is not required for the neoplastic phenotype.

Armstrong and Roman (1997) compared the ability of HPV-16 and HPV-6 E7 proteins to transactivate the AdE2 promoter, which alters the interaction between pRb and the transcription factor E2F. HPV-16 E7 had a greater effect on transactivation than did HPV-6 E7 in baby rat kidney cells and human foreskin keratinocytes. Mutation of the HPV-6 E7 to introduce the higher affinity pRb binding site of HPV-16 E7 resulted in an increase in transactivation by the HPV-6 E7. In other cell types, however, the transcriptional activation activity of high- (HPV-16) and low-risk (HPV-6) HPV E7 proteins for the Ad E2 promoter was similar (Münger *et al.* 1991).

6.3.2.3.4 Cell cycle regulation

Caldeira *et al.* (2000) compared the ability of E7 proteins from mucosal HPV-16, 32, and 54 and from cutaneous HPV-10, 48, and 77 to regulate the cell cycle in rodent immortalized fibroblasts. HPV-16, 32, 54, and 77 E7 proteins bound to proteins involved in controlling cell cycle regulation (e.g. pRb, p107, and p130, also referred to as pocket proteins), whereas the E7 proteins of HPV-10 and 48 did not. All of the HPV E7 proteins stimulated the proliferation of immortalized rodent fibroblasts, even those that did not

bind to the cell cycle-regulation proteins. The authors suggested that there was an additional mechanism other than binding to pRb or p107 by which the E7 protein promotes cell growth. Anchorage-independent growth in soft agar was observed only in NIH3T3 cells that expressed either HPV-16, 32, or 77 E7 protein, which correlates with the strength of the interaction between E7 and pRb or p107. G1/S progression and activation of cyclin E and A promoters were stimulated by all E7 proteins except HPV-10 E7. The stimulation of G1/S progression did not correlate with the pocket protein binding efficiency. The authors concluded that different E7 proteins use different mechanisms to alter cell cycle regulation and that the oncogenicity of HPV is not determined only by the ability of E7 to associate with the pocket proteins. Similar results were obtained by Helt *et al.* (2001) who reported that carboxyl-terminal sequences of E7 contribute to E7-mediated deregulation of cell cycle control.

HPV-18 E7 protein bound cyclin E and cyclin A *in vivo* and thereby became associated with the kinase cdk2 (McIntyre *et al.* 1996). Unlike the interaction of E7 with cyclin A, that of E7 with cyclin E was indirect, occurring through p107. The authors proposed that cyclin E/E7/cdk2 complexes might alter the temporal activity of cyclin/kinase, change the action of regulatory proteins such as p21, or affect p107-mediated regulation of E2F. HPV-16 E7 proteins also can interact with and inactivate the cyclin-dependent kinase inhibitors p21cip1/waf1 (Funk *et al.* 1997, Jones *et al.* 1997b) and p27kip1 (Zerfass-Thome *et al.* 1996). These inhibitors play important roles in inducing growth arrest in differentiating keratinocytes (Alani and Münger 1998) and are also activated in response to cytostatic cytokines inducing transforming growth factor (TGF)-beta (Polyak *et al.* 1994, Elbendary *et al.* 1994). Hence the ability of E7 to abrogate the activities of the cdk inhibitors may be related to the ability of E7 to retain differentiating cells in a replication-competent state (Jones *et al.* 1997b, Cheng *et al.* 1995).

6.3.2.3.5 Genomic instability

Based upon cytogenetic instability seen in human keratinocytes transfected and immortalized with full length HPV-16 DNA, Hashida and Yasumoto (1991) used plasmids that contained either the HPV-16 E6 or E7 gene to show that transfection with the E7 gene induced aneuploidy, but transfection with the E6 gene did not.

Tltsy’s laboratory showed that expression of high-risk (type 16), but not low-risk (type 6), HPV-derived E6 and E7 proteins were individually able to induce genomic instability in normal human cells that typically lack genomic instability. Interestingly, the manifestations of E6 and E7-induced genomic instability were different; E6 induced gene amplifications and deletions, whereas, E7 induced abnormal chromosome numbers, i.e., aneuploidy (Tlsty 1990, Livingstone *et al.* 1992, White *et al.* 1994).

Duensing *et al.* (2000) reported that analysis of tissue specimens from patients with HPV-associated squamous intraepithelial lesions of the cervix revealed mitoses with abnormal centrosomes. They also showed that normal human keratinocytes expressing HPV-16 E6 and E7 oncoproteins had centrosome abnormalities. Expression of HPV-6 E6 or E7 proteins individually induced abnormal chromosome numbers. While HPV-16 E7, but not E6, affected centrosome synthesis and duplication, the E6 and E7 proteins cooperated to induce centrosome-associated defects of mitotic spindle formation, including

asymmetrical arrangement of the condensed chromosomes and unaligned chromosome material, potentially resulting in aneuploidy and chromosomal instability.

Additional studies have shown that E7 can rapidly induce centrosome abnormalities prior to the detection of nuclear or chromosomal alterations. Hence E7 may act as a mitotic mutator and cause an increased incidence of abnormal mitoses, thus driving the process of genomic instability (Duensing *et al.* 2001). An analysis of centrosome abnormalities and ploidy showed an increase of both parameters during malignant progression of HPV-associated lesions (Skyldberg *et al.* 2001).

6.3.2.3.6 Immune evasion

Using the yeast two-hybrid assay, Park *et al.* (2000) showed that HPV-16 and 11 E7 proteins interacted with the interferon regulatory factor (IRF)-1, a possible tumor suppressor protein. HPV-16 and 11 E7 proteins abrogated the transactivation function of IRF-1. The HPV-16 E7 protein mediated repression of IRF-1, probably by recruiting histone deacetylase to the response region of IRF-1. Since IRF-1 may be associated with the antiproliferative effect of interferons, the authors proposed that HPV E7 protein might be part of the immune-evading mechanism relevant to cervical cancer.

6.3.2.4 HPV E6 and E7 in combination

The data concerning the transforming potential of the HPV E6 and E7 proteins have been summarized by Howley and Lowy (2001). The viral upstream regulatory region (URR), in concert with the E2 gene product and a number of cellular regulatory factors, controlled the expression of E6 and E7 in infected cells. HPV E6 and E7 immortalized human keratinocytes as efficiently as the entire early region of the viral DNA; however, neither E6 nor E7 alone was effective (Hawley-Nelson *et al.* 1989, Münger *et al.* 1989). HPV E6, alone or with E7, immortalized and transformed human breast epithelium (Band *et al.* 1991). High-risk HPV-16 E7 induced immortalization of human breast epithelial cells (Wazer *et al.* 1995) and ovarian epithelial cells (Tsao *et al.* 1995).

Kitasato *et al.* (1991) studied the ability of HPV-33 E6 and E6-E7 gene constructs to transform NIH3T3 cells as assayed by tumor formation in nude mice. While no tumorigenicity was detected for the E7 gene alone, the combination of E6 and E7 genes or E6 plus the noncoding region (NCR) was tumorigenic.

Gilles *et al.* (1993, 1994) established ten immortalized cervical keratinocyte cell lines by transfection with HPV-33 DNA. Five of these cell lines were maintained for over 100 passages with no signs of senescence. The authors reported that individual cell lines experienced four or five growth crises, which is in contrast to more efficient immortalization by HPV-16 or 18. The E6/E7 region of the virus appeared to be conserved in the HPV-33-transfected cell lines based on strong hybridization with an E6/E7 probe.

HPV E6 and E7 proteins individually have a variety of effects on cell proliferation and genetic abnormalities; however, the combination of E6 and E7 is necessary to initiate neoplastic transformation. Dexamethasone treatment of SW 756 cervical squamous carcinoma cells induced repression of E6-E7 expression and subsequently resulted in

decreased plating efficiency and decreased or no growth in soft agar (von Knebel Doeberitz *et al.* 1994). E6-E7 expression and neoplastic growth were restored upon withdrawal of dexamethasone or transfection with a plasmid containing the HPV-18 E6 and E7 genes under control of a dexamethasone-inducible repressor.

6.3.2.4.1 Studies in transgenic animals

Utilizing transgenic mice that contained a copy of HPV E6, E7, or both genes, Song *et al.* (2000) elucidated the role of each oncoprotein in the immortalization, transformation, and progression pathway. Mice that expressed the E6 protein primarily developed malignant tumors, whereas those that expressed the E7 protein usually developed benign tumors. Neither E6 nor E7 transgenic mice formed tumors when treated with the tumor promoter tetradecanoyl phorbol acetate (TPA). However, tumors did form in E7 mice after application of the initiator 7,12-dimethylbenz[a]anthracene (DMBA), indicating that the E7 protein promoted tumor formation. E6 mice treated with DMBA also developed tumors, but at a lower multiplicity than in the E7 mice. The data suggested that E7 was a promoter, but not an initiator, and that E6 acted as a weak promoter and not as an initiator. The effect of E6 on progression was determined by monitoring the papillomas that were induced in mice treated with DMBA and TPA. The papillomas converted to a malignant state earlier in the E6 mice than in the nontransgenic mice, suggesting that E6 was able to cause malignant progression. The cooperation of E6 and E7 was determined in transgenic mice that expressed both oncoproteins in the epidermis. Tumor incidence in the E6-E7 mice was significantly higher ($P < 0.001$), developed earlier, and progressed more quickly to a malignant state than in the mice expressing either E6 or E7 alone.

6.3.2.4.2 Growth factor receptors

Another possible mechanism by which the HPV oncoproteins may contribute to immortalization is through interference with growth receptors. When transfected with retroviruses encoding the HPV-16 E6 and E7 genes, cells that lacked the epidermal growth factor receptor (EGF-R) produced fewer immortal colonies than cells heterozygous or wild-type homozygous for EGF-R (Woodworth *et al.* 2000). Differences in the frequency of immortalization were not related to changes in E6/E7 RNA expression. Lack of the EGF-R resulted in decreased formation of papillomas and decreased progression of grafted cells to carcinomas. EGF-R was important, but not essential, for immortalization by HPV-16 E6 and E7 as infection with retrovirus encoding v-Ha-ras, which is part of the downstream signaling cascade for EGF-R, could induce carcinomas in cell grafts with and without EGF-R. Conversely, immortalization by E6 or E7 alone was not sufficient for progression. Caveats to these conclusions included the fact that the HPV E6 and E7 genes were overexpressed from a retrovirus long terminal repeat instead of the endogenous HPV upstream regulatory region and that overexpression of E6 and E7 may bypass the EGF-R requirement for immortalization.

6.3.3 HPV long control region (LCR)

Although the LCR does not code for a gene product, the region is important for the regulation of viral transcription and replication (reviewed by Howley and Lowy 2001). Sequences in the LCR control the transcriptional program of the papillomaviruses via transcriptional control of the E2, E5, E6, and E7 early region proteins.

In a study by Kook *et al.* (1998), LCR-driven HPV-16 promoter activity was measured in normal cells, immortalized cells, and tumorigenic cells. LCR promoter activity was higher in tumorigenic than in immortalized cells. The LCR contains six nuclear factor I (NFI) recognition sequences. Mutations in the NFI sites impaired the LCR enhancement of the P97 promoter. Kozuka *et al.* (2000) compared two forms of the LCR, one with a deletion of 38 base pairs, which eliminated a YY-1 silencer motif (Dong *et al.* 1994), and observed enhanced transcription of the HPV-16 E7 gene from the P97 promoter. Examination of biopsy tissue from 51 cervical cancer biopsies revealed two samples that contained mutations in YY-1 motifs. The mutant LCRs had augmented enhancer-promoter activity. The authors suggested that mutations affecting the YY-1 motif in the LCR may be a mechanism by which viral oncogene expression is enhanced.

A recent study (Tornesello *et al.* 2000) examined the LCR of HPV-16 isolates from penile carcinoma biopsies taken from five Ugandan patients. All five samples contained point mutations in the HPV-16 LCR; additionally, two samples had rearrangements of the LCR. LCRs from the biopsy samples were cloned into a plasmid upstream of a CAT reporter gene. The LCRs were under control of their P97 promoter, to assess their ability to regulate expression of the HPV early genes. Rearranged LCRs cloned from the biopsies showed increased levels of promoter activity; mutated and rearranged LCRs enhanced the transforming activity of the E6 and E7 genes by 1.4- and 3.0-fold, respectively. The authors suggested that increased HPV LCR activity of tumor-derived natural variants may be associated with increased E6/E7-mediated transforming activity.

6.3.4 Transcriptional modulation by HPV proteins

As described previously (Section 6.3.1), the HPV E6 and E7 proteins interact with a variety of cellular proteins. Some of the interactions result in changes in the transcription of viral and cellular genes.

The HPV-16 E2 protein bound to specific DNA sequences in the LCR upstream of the P97 early gene promoter and repressed transcription of the early region genes, E6 and E7 (reviewed by Howley and Lowy 2001). Disruption of the E2 gene by integration into the cellular DNA resulted in derepression of the viral E6 and E7 genes and enhanced immortalization of transfected human keratinocytes (Romanczuk and Howley 1992). The E2 protein of bovine papilloma virus (Wu *et al.* 2000) repressed transcription of cdc25A, a tyrosine phosphatase required for cell cycle progression in cervical carcinoma cells. E2 protein-mediated repression occurred through the transcription factor, E2F. Since HPV E7 activates cdc25 expression through an E2F-dependent mechanism (Katich *et al.* 2001), the observed repression by E2 likely represents an indirect effect mediated by repression of E7 expression through LCR sequences. Studies showed that HPV-18 E7 modulated transcription through interaction with several cellular transcription factors. HPV E7 formed a complex with pRb and activated the transcription of genes that regulate cell proliferation by releasing the transcription factor E2F from pRb complexes (Phelps *et al.* 1991). E7 also can activate expression of the c-fos gene through a cyclic-adenosine monophosphate (cAMP) response element (Morosov *et al.* 1994).

6.3.5 Changes in cytokine expression and angiogenesis

The growth of malignant tumors depends on both the removal of inhibitory mechanisms of cell proliferation and the development of a suitable local environment. The local environmental effects include suppression of immune response to tumor cells and development of a new vascular network via neoangiogenesis.

Bequet-Romero and López-Ocejo (2000) showed that HPV-16-positive epithelial cells secreted angiogenic factors into the medium, resulting in stimulation of endothelial cell proliferation. Increased levels of the potentially angiogenic cytokines bFGF, IL-8, TGF-β, TNF-α, and vascular endothelial growth factor (VEGF) were detected in HPV-positive cells. Concomitant with the increase in cytokine expression was a decrease in the expression of the angiogenesis inhibitors thrombospondin (TSP)-1 and TSP-2. Since the expression of proangiogenic cytokines was significantly increased in HPV-16-positive cells, the authors concluded tumor growth and invasion of the surrounding tissues might be supported by cervical cancer cells that express HPV-16.

López-Ocejo *et al.* (2000) studied the upregulation of the VEGF gene in immortalized keratinocyte cells (HaCaT), HPV-16-positive cervical carcinoma cells (HeLa), HPV-16-positive human keratinocytes (CaSki), HPV-16-transformed foreskin keratinocytes (HPK1A), and HPV-negative cervical cancer cells (C33A). VEGF levels were 2-fold higher in C33A cells than in the HaCaT control cell line. However, the VEGF mRNA levels were 4- to 7-fold higher in the HPV-16-positive cells (HeLa, CaSki, or HPK1A); the induction of VEGF was independent of EGF-R and TGFα activity. The HPV-16 E6 protein transactivated the VEGF gene in a p53-independent manner, suggesting that HPV-16 E6 protein may contribute to tumor angiogenesis through direct stimulation of the VEGF gene. SiHa cells (HPV-16-positive human cervical carcinoma cells) released extracellular HPV-16 E7 protein, which specifically enhanced the release of angiogenic cytokines, including TNFα, IL-1β and IL-6 by macrophages or dendritic cells (Le Buanec *et al.* 1999).

Nees *et al.* (2000) showed that TGF-β2 and TGF-β-responsive genes were downregulated in differentiating cervical keratinocytes infected with HPV-16 E6 and E7 genes. HPV-16 E6 downregulated the expression of cell-associated TGF-β2 in a p53-dependent manner while HPV-16 E7 down-regulation of TGF-β2 expression was dependent upon the pRb gene. Low-risk HPV-6b E6 and E7 did not alter either expression of cellular genes regulated by TGF-β or secretion of TGF-β2. Since loss or altered expression of TGF-β1 is thought to be an early event in cervical carcinogenesis and is frequently observed in cervical intraepithelial neoplasia, the authors proposed that HPV down-regulation of TGF-β2 may contribute to growth stimulation, immortalization, and carcinogenesis.

6.3.6 Chromosomal abnormalities in HPV-associated cancers

Changes in chromosomes 1, 2, 3, 4, 5, 6q, 7, 8q, 9, 11, 12q, 13q, 14q, 15q, 16, 17q, 18q, 19, 20q, and 22q were observed in cervical carcinomas (Lazo 1999, Hidalgo *et al.* 2000). A gain in chromosome 3q was detected in 7.7% (1 of 13) of cervical dysplasias and 90% (9 of 10) of invasive cervical carcinomas, in which HPV-16, 31, 33, 45, and 58 were

consistently detected (Heselmeyer *et al.* 1996). The authors proposed that the gain of chromosome 3q is an important event in the transition of dysplasia to invasive cervical carcinoma. Examination of 24 HPV-positive (16, 18, or 33) cervical carcinomas (12 primary tumors and 12 tumor-derived cell lines) revealed a high incidence of LOH for chromosome 3p14 (Hidalgo *et al.* 2000). In a 1996 review by zur Hausen, the most frequent LOH were observed for chromosomes 3p13-25, 6p21-23, and 18q12-21. Mitra (1999) showed that 64% of cervical cancers with LOH for chromosome 3p also were positive for high-risk HPV-16 or 18, while 79% of cervical cancers that showed LOH for chromosome 5p were negative for high-risk HPV. The chromosome 3p region has been implicated in a number of cancers, including breast cancer, lung cancer, ovarian cancer, renal cell carcinoma, and testicular cancer (Lazo 1999).

Microsatellite analysis was performed with three chromosome 3p markers to determine allele loss in 22 LSIL and 15 high-grade HSIL of the cervix (Chu *et al.* 1999). Allelic loss of any of the 3p markers was seen in 23% of the LSIL and 27% of the HSIL, while microsatellite alterations occurred in 41% of the LSIL and 67% of HSIL. No correlation was found between HPV infection (types 16, 18, 31, 52, 58, and 68) and microsatellite alterations or allelic loss; however, only a subset of cases were genotyped.

Cervical cancer cells and HPV-16-immortalized cervical or foreskin keratinocyte cells were analyzed to identify karyotypic changes (Cottage *et al.* 2001). Spectral karyotyping and multiplex-fluorescence *in situ* hybridization (FISH) analysis revealed that all cell lines examined contained chromosomal aberrations, and a translocation of chromosome 10q was observed in 9 of the 12 cell lines. The authors noted that the sequences across the pericentric region of chromosome 10 have undergone duplication and contain many zinc finger transcription factor-encoding genes. Additionally, these sequences may be disrupted in HPV-immortalized cells, resulting in transcriptional changes in cellular and viral gene expression. However, it is not clear that such chromosomal aberrations also commonly occur in HPV-positive cancers.

6.4 HPV in transgenic mice

Transgenic mice have been a useful tool in delineating the role(s) that HPV proteins play in the development of hyperplasia and dysplasia as well as in the progression to benign and malignant tumors.

6.4.1 HPV-transgenic mice with the HPV control region

Searle *et al.* (1994) developed two lines of transgenic mice using a plasmid that contained the HPV-16 DNA with the noncoding URR. Neither line expressed the transgene. Replacement of the URR with the bovine keratin 6 (bK6) gene, however, resulted in transgenic animals with HPV gene expression.

The HPV-18 LCR, along with the E6 gene, E7 gene, and a large part of E1, was introduced into mice (Comerford *et al.* 1995). Founder males exhibited enlarged preputial glands and seminal vesicles by 50 weeks of age, while similar changes were observed in 81% of hemizygous and 93% of homozygous male progeny by 12 weeks of age. Enlargement was caused by polyploid hyperplasia of the secretory epithelium and by

fluid distension. Viral proteins E6 and E7 were expressed only in the seminal vesicles, preputial gland, and kidneys. Approximately 41% of the transgenic females developed cervical neoplasms at 1 to 2 years of age. The tumors that formed in female mice were mesenchymal in origin and not epithelial like the cervical carcinomas seen in humans. The authors suggested that in HPV-18 LCR-transgenic mice, expression of HPV oncoproteins was directed to the urogenital tract, most likely by an element present in the LCR.

6.4.2 Transgenic animals with epithelial cell-specific expression of the HPV oncogenes

Regulatory elements of the human keratin K1 (HK1) gene were used to target the expression of HPV-18 E6 and E7 to the epidermis in mice (Greenhalgh *et al.* 1994). Small lesions and rare, spontaneous, c-rasHa-expressing papillomas were observed in 1-year-old (or older) transgenic mice. Newborn epidermis expressed the E6 and E7 HPV oncoproteins but with a predominance of the truncated E6, E7 transcript. The low level of intact E6 transcripts may explain the long latency period. Alternatively, the long latency may suggest a requirement for additional cellular events for lesion production.

Searle *et al.* (1994) created transgenic mice that contained the entire HPV-16 early region under control of transcription regulatory sequences from the bovine keratin 6 gene. Eight transgenic lines were developed, and malignant carcinoids of the stomach were found in mice from all eight independent lines. At 200 to 400 days of age, the incidence of stomach tumors reached 100%. The average age of tumor presentation was consistently lower in male mice than in females. HPV mRNA was detected in the tongue, forestomach, glandular stomach, female reproductive tract, and tail skin of the transgenic mice. Studies of the gastric mucosa revealed progression from dysplasia to extensive colonization with dysplastic cells, and finally to metastatic tumors. The tumors appeared to develop from the glandular region of the stomach and contained increased levels of HPV E6 and E7 mRNAs. The delayed appearance and focal origin of the tumors suggested that additional events were necessary for neoplasia.

Auewarakul *et al.* (1994) used the bovine cytokeratin 10 (K10) promoter to target expression of the HPV-16 E6 and E7 genes to the suprabasal layers of the epidermis in transgenic mice. Increased basal cell proliferation and suprabasal mitotic activity were observed in the epidermis of skin and forestomach of heterozygous transgenic mice compared to nontransgenic controls. TGF-α and c-myc were overexpressed in transgenic skin. Treatment with TGF-β prevented TPA induction of c-myc in transgenic but not in nontransgenic mice, suggesting that the activity of TGF-β may be inhibited in the skin of HPV-transgenic mice.

Using a human keratin 14 (K14) enhancer/promoter, Arbeit *et al.* (1994) targeted expression of the HPV-16 early region to the squamous epithelium in transgenic mice. The E6 and E7 genes were used in combination with either wildtype or mutant E1 and E2 genes that abolished their function. The absence of E1 or E2 function had no effect on the phenotypes of the transgenic mice. The ear epidermis had some degree of hyperplasia in every mouse. Progressive squamous epithelial neoplasia was observed at multiple epidermal and squamous mucosal sites including truncal skin, face, snout, eyelids, and

anus. Discernible stages of progression were part of the phenotype; mild hyperplasia progressed to hyperplasia and resulted in dysplasia and papillomatosis. Chronic treatment of these animals with small doses of estrogen induced SCC of the cervix and vulva. In untreated transgenic animals, HPV oncogene expression was detected only during estrus, but estrogen-treated animals showed persistent expression of HPV oncogene (Arbeit *et al.* 1994, Elson *et al.* 2000)

In a different mouse strain, expression of the isolated HPV-16 E6/E7 genes from the K14 promoter yielded a high incidence of SCC in the skin (Lambert *et al.* 1993). Expression of the individual genes E6 or E7 from the K14 promoter also resulted in tumor formation, albeit at a lower level (Herber *et al.* 1996). Skin painting experiments with chemical carcinogens suggested that E6 and E7 play different and cooperating roles during tumor formation (Song *et al.* 2000).

6.5 Cofactors in HPV oncogenesis

The cofactors described below also are discussed in Section 3.5.2.

6.5.1 Other viruses

A meta-analysis by Mandelblatt *et al.* (1999) showed an interaction between HPV and HIV, with a strong association between HPV and cervical neoplasia. Two potential mechanisms proposed by the authors for the HPV-HIV interaction are: 1) HIV may affect immune function, which could increase susceptibility to HPV and oncogenicity; or 2) a direct molecular interaction may occur between HPV and HIV.

Vernon *et al.* (1993) reported a direct interaction between HPV and HIV in which the HIV-1 tat protein enhanced the transcription of HPV-16. Tat1 also can stimulate growth of cells, inhibit T-cell proliferation, transactivate various viral promoters, and interact with the upstream regulatory region of HPV-16. However, Wright and Sun (1996) noted that HIV and HPV viruses do not coinfect cervical epithelial cells or colocalize in cervical tissues. Any interactions between the two viruses are likely to be mediated by extracellular factors.

6.5.2 Chemicals

Numerous studies have shown that bracken fern, a plant that contains mutagens, carcinogens, and immunosuppressants, is a cofactor for BPV-4 carcinogenesis in cattle (Jackson *et al.* 1993). Both BPV-4 and bracken fern were involved in bladder carcinogenesis, and the immunosuppression caused by the ingestion of bracken fern may have resulted in activation of latent BPV-4. Campo *et al.* (1992, 1994) showed that full malignant progression of such viral lesions also required the chemical mutagens and carcinogens contained in the bracken fern (see Section 4.2.1). The role of immunosuppression in HPV-associated cancers is discussed in Section 6.6.1.

Evidence for cooperation of HPV and chemicals in carcinogenesis has been provided by cell and animal studies. Two studies (Sizemore *et al.* 1995, Rorke *et al.* 1998) showed that the formation and removal of polycyclic aromatic hydrocarbon (PAH) adducts,

resulting from exposure to B[a]P, benzanthracene, or 3-methylcholanthrene, were similar in normal ectocervical cells and in HPV-16-immortalized cervical cells. PAH-exposed, HPV-immortalized cervical cells showed diminished growth inhibition and decreased differentiation. HPV infection impaired the response to the genotoxicant and increased the risk of mutation in HPV-immortalized cells. After treatment with PAH, HPV-immortalized cervical cells showed a smaller induction of p53 than normal cervical epithelial cells. Consequently, the immortalized cells were able to proliferate even though they were genetically damaged and were at increased risk for the formation of mutations. Comparable chemical cofactor effects were seen in mice inoculated with NHOK, HOK-18 (NHOK transformed with HPV-18), or HOK-18 cells exposed to MNNG *in vitro* (Shin *et al.* 1994). Tumors developed only in mice that received MNNG-treated HOK-18 cells.

6.5.3 Hormones

Estradiol and the estrogen metabolite 16α-hydroxyestrone promoted anchorage-independent growth in HPV-16-immortalized keratinocytes (Newfield *et al.* 1998). The authors concluded that the malignant phenotype of HPV-immortalized cells may be increased by exposure to the estrogen metabolite, 16α-hydroxyestrone.

As discussed in Section 6.4.2, Arbeit *et al.* (1996) demonstrated synergistic cooperation between chronic estrogen exposure and HPV-16 oncogenes that resulted in gynecologic pathology in female mice. Michelin *et al.* (1997) utilized transgenic mice that carried the α-galactosidase gene under control of the HPV-18-URR promoter as a model system to examine the effects of pregnancy and steroid treatment on URR activation. Pregnancy resulted in a slight decrease, followed by an increase in β-galactosidase activity. To test the effects of sex steroids on the URR, ovariectomized transgenic mice were implanted with hormone pellets that contained either progesterone or estradiol. Exposure to either hormone resulted in an increase in β-galactosidase activity. The authors suggested that progesterone and estradiol activated the HPV-URR promoter and postulated that the interaction between the viral URR and sex steroids could have clinical implications in women infected with high-risk HPVs.

HPV-negative human cervical cancer cells (C33A), human cervical cancer cells with integrated HPV-16 DNA (CaSki), human laryngeal carcinoma cells transfected with an HPV-16 expression vector (V16-Hep-2), and nontransfected human laryngeal carcinoma cells were exposed to progesterone, and changes in proliferation and the ability to form colonies in soft agar were noted (Yuan *et al.* 1999). Exposure to progesterone resulted in significantly greater colony formation only in the HPV-positive cells, i.e., CaSki cells and V16-Hep-2 cells (Student's t test, $P < 0.01$). Progesterone slowly increased the level of E6 and E7 transcripts by delaying the degradation of the transcripts. The authors concluded that the growth properties of malignant epithelial cells containing HPV-16 DNA were enhanced by progesterone and that progesterone increases the expression of HPV-16 E6 and E7 at least partially by decreased degradation of the transcripts.

6.5.4 Radiation

HPV oncoproteins have been linked to decreased repair of UV-induced DNA damage. After UV exposure, HPV-16-immortalized human oral keratinocytes exhibited a delayed onset and a lower level of nucleotide excision repair (NER) than normal human oral keratinocytes (Rey *et al.* 1999). UV-exposed human fibroblasts that expressed the HPV-16 E7 oncoprotein were defective in global NER but proficient in transcription-coupled NER, suggesting a potential role for E7 in UV-associated skin cancer (Therrien *et al.* 1999).

Santin *et al.* (1998) showed that doses of gamma-radiation (1,250 to 10,000 cGy) increased the expression of viral HPV-16 E6/E7 oncogenes and major histocompatibility complex (MHC) Class I restriction elements in HPV-16-infected cervical carcinoma cells (SiHa and CaSki). The authors proposed that the enhanced tumor immunogenicity that resulted from the up-regulation of the MHC class I restriction elements could be offset by the up-regulation of E6/E7, which conferred a growth advantage to radiation-resistant cells. Song *et al.* (1998) observed similar results *in vivo*. Transgenic mice that expressed HPV-16 E6 or E7 genes in the epidermis were exposed to ionizing radiation. Labeling with bromodeoxyuridine revealed no inhibition of DNA synthesis in cells obtained from the gamma-radiation-exposed transgenic mice, although nontransgenic mice showed almost complete arrest of the cell cycle after a similar exposure to radiation. The abrogation of DNA synthesis inhibition was more complete than that induced by loss of p53.

6.6 Immune mechanisms and HPV-associated neoplasia

6.6.1 Immunosuppression

Previously, experiments showed that cattle fed a diet of bracken fern were immunosuppressed (see Section 4.2.1) as evidenced by a dramatic fall in polymorphonuclear leukocytes and a chronic drop in circulating lymphocytes (Campo *et al.* 1992). The immunosuppression induced by the bracken fern diet resulted in reactivation of latent virus in BPV-4-inoculated animals and in those naturally infected with BPV-4. BPV-4-infected, immunosuppressed animals developed bladder cancer. In further studies, Campo *et al.* (1994) showed that animals immunosuppressed by chemicals in the bracken fern and subsequently infected with BPV-4 exhibited spreading of papillomas. The papillomas persisted beyond the average papilloma lifespan observed in immunocompetent animals. The authors concluded that the immunosuppression appeared to be necessary but not sufficient for neoplastic progression, which is dependent on other chemical components of the bracken fern (see Section 6.5.2).

The prevalence of oral warts in the immunosuppressed population has been reported to be 3- to 20-fold higher than that in the general population (Leigh *et al.* 1999). Oral warts found in immunodeficient patients were generally different in morphology and contained unusual HPV types. The high incidence of cutaneous warts, genital warts, and anogenital intraepithelial neoplasia among patients with genetic, iatrogenic, or acquired immune deficiencies (AIDS) suggested that immune factors may play a role in the control of certain HPV infections (Favre *et al.* 1997).

Lesions from immunosuppressed (renal transplant) individuals had a high prevalence of HPV DNA from 7 cutaneous, 3 mucosal, and 21 EV HPV types, as detected by PCR with degenerate and type-specific HPV DNA primers (Harwood *et al.* 2000). The three mucosal HPV types were HPV 11, 16, and 66. The HPV DNA prevalence was significantly lower ($P < 0.001$, $\chi 2$ test) in immunocompetent individuals. Similarly, Vernon *et al.* (1995) showed that there was a greater prevalence of HPV infection in HIV-infected individuals, and the increase was proportional to the severity of immunosuppression. Coinfection increased the risk for genital intraepithelial neoplasia, and this increase in risk reflected the severity of immunosuppression. Women with reduced immunocompetency (transplant patients, pregnant women, and women with AIDS) had increased incidence of HPV infection and cervical neoplasia (Mandelblatt *et al.* 1999).

Heard *et al.* (2000) suggested that the transition from high-load HPV infection to cervical disease might depend on the level of local immune surveillance, among other factors. Low HPV-DNA load was a risk factor only in severely immunosuppressed women. Cervical high-load HPV infection and clinical expression of HPV in HIV-seropositive women were increased by immunosuppression. In HIV-positive women, there was a significant interaction between CD4 cell counts and the detection of oncogenic HPV types. HPV was detected more frequently in HIV-seropositive women than in HIV-seronegative women (Wright and Sun 1996).

Palefsky and Barrasso (1996) comprehensively reviewed HPV infection and disease in men. The risk of HPV-related anal disease was increased in immunosuppressed persons, regardless of sexual orientation. The data suggested that most, if not all, HIV-positive homosexual and bisexual men had anal HPV infection that was most commonly associated with HPV type 16, and the rate of anal disease varied inversely with the CD4 level.

6.6.2 Cell-mediated immunity

Tindle and Frazer (1994) summarized numerous studies that showed evidence for the involvement of cell-mediated immunity, including inflammatory infiltrate in skin warts and in genital HPV infections. In general, intraepithelial lymphocytes and Langerhans cells were depleted, while the number of natural killer cell markers increased. Spontaneous regression of warts was associated with lymphocyte and macrophage infiltration.

Cell-mediated immunity probably plays a major role in HPV infection control (Favre *et al.* 1997). Regressing flat and genital warts contained infiltrates of lymphocytes and macrophages. Other research showed that cytotoxic T lymphocytes specific to tumor cells expressing HPV E6 or E7 oncoproteins were induced in mice. HPV-16 E6 and E7 proteins have human cytotoxic Tlymphocyte epitopes. Human cytotoxic T-lymphocyte epitopes in HPV-16 E6 and E7 have been identified.

Nakagawa *et al.* (2000b) showed that lack of cytotoxic T-lymphocyte response to HPV-16 E6 antigens was important in the persistence of HPV-16. However, lack of response to HPV-16 E7 protein appeared to be unimportant.

Cervical carcinoma patients and cervical neoplasia patients provided cervical scrapes for HPV genotyping and blood for the measurement of HPV-specific cytotoxic T lymphocytes (Bontkes *et al.* 2000). The presence of memory cytotoxic T lymphocyte precursors (mCTLp) specific for HPV-16 E6 and E7 was associated with the persistence of HPV-16 virus. Patients who had cleared the virus did not have detectable levels of HPV-specific mCTLp. The authors noted that the presence of HPV-specific mCTLp did not appear to prevent the development of a high-grade lesion although the results did not exclude the possibility that vaccine-induced CTL might be therapeutic.

6.6.3 Major histocompatibility complex (MHC) expression

Cromme *et al.* (1993) analyzed CIN lesions and cervical carcinomas for MHC I and MHC II expression by immunohistochemistry, Aberrant MHC I expression was observed both in HPV-negative lesions and in lesions that contained high-risk (HPV-16) HPV. Up-regulation of MHC II did not correlate with HPV infection. The presence of HPV E7 transcripts was not related to MHC I expression changes in CIN lesions. The authors concluded that variations in MHC I expression were not directly correlated with the presence of specific HPV types. MHC I and II alterations were present in cervical intraepithelial neoplasia independent of the presence of HPV DNA.

The expression of MHC class I antigens was reduced or absent in a majority of CIN lesions and cervical carcinomas (Wu 1994). MHC class II antigens were expressed in 50% of high-grade CIN lesions and in approximately 90% of cervical cancers. One study showed that expression of MHC class I molecules was greater in HPV-16-positive dysplastic lesions than in HPV-16-negative tissue from the same patient. Generally, the pattern of expression of MHC molecules was independent of the presence of HPV lesions in the cervix and vulva.

HLA class I and class II expression (separately or together) was frequently altered in SCCs of the cervix. A large proportion of cervical carcinomas was positive for high-risk HPV DNA, but there was no association between HLA class I and II changes and HPV DNA. Similarly, change in HLA I expression in cervical premalignant lesions was not associated with the presence of high-risk HPV (types 16, 18, 31, 33) DNA (Stern and Duggan-Keen 1994).

6.6.4 HLA polymorphisms: Association with cervical cancer risk

The presentation of peptides to the immune system is controlled, at least in part, by the expression of HLA subtypes. An increased frequency of some HLA genotypes in patients with cervical neoplasia compared to the normal population suggests that inherent genetic factors may control the immune response to HPV (see section 3.5.1). In addition, some class I HLA antigens are down-regulated in both preinvasive and invasive lesions

associated with HPV. Very little original research on the relationship between HPV and HLA haplotypes has been published since the IARC meeting in 1995.

6.7 Summary

High-risk HPV-16 and 18 can immortalize and transform cells. These two viruses are consistently expressed in HPV-positive cancer after the integration of the HPV genome. Although other viral proteins also play a role in HPV-induced transformation, the primary immortalization and transformation proteins are E6 and E7. Studies with transgenic mice further support the notion that E6 and E7 proteins are important in HPV-associated neoplasia. The E6 protein interacts with p53 by increasing the degradation of p53, thereby interfering with apoptosis. The E7 protein disrupted transcription factor complexes with pRb and related cell cycle control proteins, altering transcriptional controls and cell cycle progression. Both the E6 and E7 proteins alter growth regulatory pathways by interfering with growth receptors or growth factors; cytokine expression was altered in HPV-16-positive cells. Expression of E7 causes mitotic abnormalities by inducing aberrant centrosome duplication. Integration of HPV-16 or 18 resulted in increased expression of the HPV E6 and E7 oncogenes. A gain in chromosome 3q was noted in cervical carcinomas containing HPV-16, 31, 33, 45, and 58. HIV, chemicals, hormones (estradiol or progesterone), and radiation may be cofactors for HPV carcinogenicity. The risk of HPV-related disease was much greater in immunosuppressed populations. Cell-mediated immunity appeared to play a role in HPV infections although it was not clear if cytotoxic T lymphocytes can prevent HPV-induced lesions. The human cancer and mechanistic data for high-risk HPVs are summarized in Table 6-2.

Table 6-2. Summary of data for high-risk HPVs

	Human Studies			
HPV type	**Case-control**	**Cohort**	**Cell culture**	**Biochemical**
HPV-16	High-risk estimates	Significant elevated RR observed for high-grade SIL carcinoma *in situ* and invasive cancer	Recombinant DNA immortalizes human foreskin keratinocytes and other cells, and E7 protein promotes anchorage-independent growth *in vitro*	E6 oncoprotein promotes degradation of p53 E7 protein inactivates cell-cycle regulatory proteins (pRb, p21, p27, p107, and p130)
HPV-18	High-risk estimates	Limited number of studies, significant risks for high-grade SIL and carcinoma *in situ*	Recombinant DNA immortalizes human foreskin keratinocytes and other cells	E6 oncoprotein promotes degradation of p53 E7 protein inactivates cell-cycle regulatory proteins
Other (not 16 & 18) high-risk HPVs – individuals	High-risk estimates for 31, 33, 35, 39, 45, 51, 52, 58, 59	Elevated non-significant risks for high-grade SIL (31, 52, 55)	Recombinant DNA (HPV-31 and 33) immortalizes human foreskin and cervical keratinocytes, E6 or E6/E7 genes (HPV-33) transform NIH3T3 cells, and E7 protein (HPV-32 and 77) promotes anchorage-independent growth *in vitro*	E7 protein (HPV-32, 54, and 77) binds to cell-cycle regulatory proteins (pRb, p107, and p130)
High-risk HPVs as a class (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68)	High-risk estimates	Significant elevated risk for high-grade SIL and CIN III	NA	NA

NA = no data available.

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Appendix A. Supporting Data: Human Cancer Studies

Table A-1. Current case-control studies and cross-sectional studies

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
DNA				
Multiple stages of CIN including invasive cancer				
Lorincz *et al.* 1992 USA - multiple sites (Michigan, Washington, D.C., and other sites)	2,627 women from 8 studies (case-series, case-control, and cohort) from 1982–1989 Normal cervix 1,566 Atypia 270 Definite CIN 791 Cancer 153 High-grade 261 Low-grade 377 HSIL – high grade SIL LSIL – low-grade SIL	HPV DNA Detected and typed using Southern blot hybridization HPV prevalence 79/6.4 (all CIN) Low risk (LR): 6/11, 42, 43, 44 Intermediate risk (IR): 31, 33, 35, 51, 52 High risk (HR): Defined as a result of study as 16, 18, 45, 56	ORs for cervical disease 27.1 (atypia as cases) and 38.7 (atypia as controls); both $P < 0.0001$ RRs for invasive cancer LR 0 IR 31 (19–52) 16 260 (217–312) 18, 45, 56 296 (199–441) RR for squamous intraepithelial lesion (SIL) HSIL / LSIL LR 24 (13–43) / 53 (36–77) IR 72 (51–102) / 22 (18–26) 16 236 (199–280) / 37 (25–55) 18, 45, 56 65 (50–85) / 33 (19–56)	HPV-58 added to list of IR based on higher frequency in cancer but not included in overall OR because all samples were not available for confirmation

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Ferrera *et al.* 1999a Honduras	Population case-control study (1993–1995) Cases: 229 women from hospitals and clinics, with histologically verified CIN and invasive cancer (ICC): 44 CIN I, 36 CIN II, 45 CIN III and 104 ICC Controls: 438 women with normal cytology, matched for age (2 per case) and clinic	HPV DNA Detected from cervical scrapes by PCR using general primers (MY11/MY09) and typed by sequencing HPV prevalence 80/39 (ICC/Control) 16-related: 16, 31, 33, 35, 52, 58 18-related: 18, 45, 59 Others: 6, 11, 21, 22, 53, 55, 56, 62, 66, 70	Crude ORs for HPV and CIN and cancer HPV — 16-related — 18-related CIN III — 47.9 (6.2–373) — 24.3 (2.4–242) ICC — 13.3 (6.0–29.3) — 23.7 (6.1–92.6) ORs for other HPV < 1.0 except for CIN III (15.2, 95% CI = 1.5–159) and adjusted OR for ICC (1.4, 95% CI = 0.2–8.0); crude OR for ICC = 0.7	OR also adjusted (not given in table) by age of first intercourse (CIN I), schooling (CIN II), and schooling and previous cytology (ICC); adjusting did not affect any ORs except for 18-related and ICC (adjusted OR = 75)
Chan *et al.* 1999 Hong Kong, China	Cross–sectional study 332 women referred to and diagnosed at hospital colposcopy clinic Cases: 51 CIN I, 29 CIN II, 60 CIN III, 16 squamous cell carcinoma (SCC), and 2 adenocarcinoma *in situ* (ACIS) Controls: 86 women with normal or inflamed cervices; 88 women with condyloma	HPV DNA Detected by PCR assay using MY09/MY11 primers and typed using restriction enzyme digest HPV prevalence Normal 19 Condyloma 36 CIN I 65 CIN II 38 CIN III 68 Carcinoma 78	Crude ORs for CIN/carcinoma HPV-16 7.4 (2.4–25.4) HPV-58 3.7 (1.2–13.0) Single infection 4.3 (1.0–18.0) (Reference double infection) Adjusted OR for CIN/carcinoma HPV-58 4 .0 (1.2–14.4) Trend for severity of cervical lesion χ^2 HPV-16 22.5, $P < 0.001$ HPV-58 5.84, $P = 0.016$	Genotyping by restriction enzymes may have difficulties identifying multiple infections with more than two types of HPV

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Herrero *et al.* 2000 Costa Rica	Population-based study (1993–1994) of a cohort of 9,175 randomly chosen women screened for cervical abnormalities; 3,024 were tested for HPV DNA, results available for 2,974 Cases: 34 women with invasive cancer, 125 HSIL, 181 LSIL (confirmed), 326 other LSIL, 698 ASCUS Controls: 1,610 women without cervical abnormalities	HPV DNA Detected by PCR using MY09/MY11 primers and typed (more than 40 types) by hybridization using type-specific probes Cancer associated strains: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 *Noncancer-associated strains: All other types, including recognized noncancer types (2, 6, 11, 32, 40, 42, 57) and undetermined oncogenic potential (53, 54, 61, 62, 64, 67, 69, 70, 72, 73, etc.) HPV prevalence Control 16 LSIL 73 HSIL 89 Cancer 88	Age-adjusted ORs and AF (attributable fraction) for HPV types and cervical neoplasia OR / AF (%) HSIL 16: 320 (97–1,000) / 45 18, 31, or 45: 56 (18–180) / 12 Other cancer: 31 (14–70) / 22 Noncancer*: 5.4 (1.8–16) / 4.0 Uncharacterized: 12 (3.4–44) / 3.6 Cancer 16: 710 (110–4,500) / 47 18, 31, or 45: 150 (22–1,000) / 15 Other cancer: 20 (4.5–90) / 17 Noncancer*: 2.2 (0.2–23 / 1.6 Uncharacterized: 27 (3.5–210) / 5.7 ORs for HSIL and cancer combined and single HPV-16 or multiple infection (with or without HPV-16) Multiple / HPV-16 (-) / HPV-16 (+) No / 1.0 (ref) / 450 (100–2000) Yes / 29 (13–66) / 190 (39–920)	Greater than 90% participation rate, allowing for populationwide estimates by sampling HPV-16 was the most common type in cancer and HSIL, HPV-18 was the second most common type found in cancer, and HPV-58 was the second most common type found in HSIL

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Sasagawa *et al.* 2001 Hokuriku area, Japan	Hospital cancer screening program (1995–1999) Cases: 366 cytologically abnormal women (145 LSIL, 137 HSIL, 72 cervical SCC, 12 cervical adenocarcinoma (ADC), and 16 condyloma acuminata) Controls: 1,562 women randomly chosen from same population as cases, with no past or current evidence of CIN or symptoms of sexually transmitted diseases	HPV DNA Detected by PCR assay using degenerate primers and typed by hybridization to HPV-specific probes	HPVs significantly (by OR) associated with CIN LSIL HPV-**11**, 16, 31, 35, **39, 42**, **44**, 51, 52, **53**, 56, 58, **59, 62, 66**, and multiple HSIL HPV-16, 31, **33, 35**, 51, 52, **56**, 58 and multiple SCC HPV-**16, 18, 31, 51, 52, 58**, and multiple ADC HPV-16, 18, and multiple High-risk types considered to be 16, 18, 51, 52, 58, and perhaps 33, 35, 56 Condyloma tissues – only HPV-6 and 11 found {Bold indicates HPV types that authors considered to be associated with a specific CIN stage}	HPV types that had a significant OR but did not occur as a single HPV type were excluded from list. Risks of HPV-6, 30, 54, 55, 61, 67, 58, 70, 72, and 73 not determined in study

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Thomas *et al.* 2001a, 2001b Bangkok, Thailand 2001a – carcinoma *in situ* 2001b – invasive cancer	Hospital case-control study (1991–1993) Also, case-case study (*in situ* and invasive cancer) Cases: 190 women with invasive SCC and 42 women with invasive adenomatous (adenocarcinomas and adenosquamous carcinomas, ADC) and 75 with carcinoma *in situ* (65 with matched controls for case-control study) Controls: 291 women from otolaryngology and surgery wards without conditions associated with the use of steroid contraceptives were matched on age and residence	HPV DNA Detected by PCR using MY09/11 primers and typed by hybridization to type-specific primers (6, 11, 16, 18, 31, 33, 35, 39, and 45) HPV prevalence SCC/ADC/controls 16 60/33/2 18 14/43/0 31/33/35/39 13/7/1 45 0/2/0 Oncogenic 79/76/3 Untyped 7/2/4	Age adjusted ORs for invasive cervical cancer SCC / ADC 16 83 (39–232) / 24 (8.7–76) 18 ∞ (22–∞) / ∞ (165–∞) 31/33/35/39 14 (4.9–61) / 7.1 (1.2–44) Oncogenic 155 (72–385) / 106 (41–317) Adjusted ORs for carcinoma *in situ* (case/control) 16 11 (3.9–33) 18 10 (1.2–86) 31/33/35/39 17 (2.2–135) Oncogenic 16 (5.9–47) Stratified analysis: Significant association of HPV (16 and/or 18) and invasive cancer in the following strata: early age of first intercourse, HSV-2 seropositive (borderline, 16 only), higher number of pregnancies, spontaneous abortion, never used an IUD, never had a cervical smear, no education, and never had a chest exam Age adjusted ORs for case/case study (*in situ* and invasive SCC and HPV 16 4.2 (1.8–9.5) 18 4.0 (1.2–13) 31/33/35/39 2.1 (0.8–5.9) Oncogenic 3.5 (1.6–7.7) Untyped 0.7 (0.3–2.1) Case/case study Significant associations for invasive cancer compared to *in situ* after adjusting for HPV were found for not having a tubal ligation, not having a cervical smear, and not having a chest exam	No subjects positive for HPV-6 or 11 Cofactors considered in stratified analysis of HPV in case-control study and used in multivariate analyses in case/case study: number of sexual partners, age at first intercourse, serology (HSV-2 syphilis, HBs, HBc), HBsAg, number of pregnancies, stillbirth, abortions (spontaneous, induced), ever used oral contraceptives, ever used DMPA, IUD, tubal ligation, cervical smears, smoking, alcohol, chest exam, and education

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
IARC-inititaled studies on invasive cervical cancer				
Chaouki *et al.* 1998 Rabat, Morocco	Hospital case-control study (1991–1993) Cases: 214 women with invasive cervical cancer, 152 with SCC, 16 with adenocarcinoma/adeno-squamous (ADC/ADS), and 46 cervical cancer without other specification Controls: 203 women from same or nearby hospital matched on age (distribution) but with no history of hysterectomy or diagnosis related to established or suspected risk factors of cervical cancer	HPV DNA Detected from biopsies of cases and exfoliated cells of controls by PCR-based assay using GP5+/6+ primers, typed using Southern analysis or hybridization with HPV-specific probe HPV prevalence Any 95/21 58 68/4 18 8/1 30s 4/1 45 5/2 50s 3/2	Age-adjusted ORs for cervical cancer 16 202 (76–533) 18 93 (18–478) 30s (31,33,35) 39 (7–226) 45 55 (12–249) 50s (51,52,56,58,59) 17 (4–75) Single 59.8 (28.2–127) Double 90.8 (17.8–464)* * OR for single versus double = 1.4 (0.3–6.7) SCC / ADC/ADS 16 220 (76–640) / 182 (16–∞) 18 94 (17–522) / 40 (2–917) 45 76 (15–391) Risk factors for cervical cancer among HPV-positive women Low family income, parity, oral contraceptive use, never had a Pap smear, and number of sexual partners before age 20	ORs for risk factors in HPV-positive strata adjusted for age and all risk factors considered, including residence, family income, number of pregnancies, oral contraceptive use, Pap smear, and number of sexual partners before age 20

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Chichareon *et al.* 1998 Thailand	Hospital case-control study (1990–1993), same matched design as Chaouki *et al.* (1998) Cases: 377 cases of incident invasive cervical cancer cases (338 with SCC and 39 with ADC/ADS Controls: 261 women from same hospital	HPV DNA Detected and typed by PCR and Southern blot as described by Chaouki *et al.* 1998 HPV prevalence Controls/ exfoliated cells 16 SCC/exfoliated cells 83 SCC/biopsy + exfoliated cells 95 ADC/ADS exfoliated cells 84 ADC/ADS biopsy + exfoliated cells 90	Adjusted ORs for cancer SCC / ADC/ADS 16: 227 (103–497) / 63 (17–232) 18: 115 (45–299) / 278 (50–1,535) 31: 44 (9–221) 33: 70 (7–696) 45: 20 (5–89) / 16 (1.2–228) 52: ∞ (31–∞) 58: 66 (13–335) 59: ∞ (15–∞) Multiple: 49 (13-184) / 30 (3-280) Risk factors for SCC and/or ADC/ADS cervical cancer among HPV-positive women were history of sexually transmitted disease, lifetime number of sexual partners, and smoking (SCC only)	ORs may have been higher than they would have been if calculated using data when HPV was measured from cases using only exfoliated cells (not biopsy) ORs for risk factors in HPV-positive strata adjusted for same factors as Chaouki *et al.* (1998) (except for residence and with the addition of education and smoking)

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Ngelangel *et al.* 1998 The Philippines	Hospital-based case-control study (1991–1993) Same matched design as Chaouki *et al.* (1998) Cases: 356 incident cases of invasive cervical cancer (323 SCC, 33 ADC) Controls: 381women women from the outpatient clinics or ward of the same hospital	HPV DNA Detected and typed by PCR and Southern blot as described by Chaouki *et al.* (1998) HPV prevalence SCC 94 ADC/ADS 91 Controls 9	Age-adjusted ORs for cancer SCC / ADC/ADS 16: 506 (178-1,436) / 549 (44–6,912) 18: 276 (99–771) / 948 (97–9,240) 45: 124 (46–-335) / 259 (26–2,618) 51: ∞ (45–∞) 52: 93 (18–484) 58: 81 (14–480) 59: ∞ (19–∞) 66: ∞ (14–∞)* Multiple: 91 (31–211) / 140 (23–973) * 66 only detected with coinfection with another high-risk HPV Risk factors for SCC and/or ADC/ADS cervical cancer among HPV-positive women: parity (both), interval since last Pap smear (SCC), and socioeconomics assessed by household amenities (ADC/ADS)	ORs for risk factors in HPV-positive strata adjusted for same factors as Chaouki *et al.* (1998) (except residence and with the addition of smoking)
Rolón *et al.* 2000 Paraguay	Hospital case-control study (1988–1990) Same matched design as Chaouki *et al.* (1998) Cases: 116 women with invasive cervical cancer Controls: 101 women from same hospital	HPV DNA Detected and typed by PCR and Southern blot as described by Chaouki *et al.* (1998) HPV prevalence 97/20	Age-adjusted ORs for cervical cancer 16: 910 (84–9,755) 18: ∞ (4–∞) 31: 110 (8–1,510) 33: 261 (17–4,090) 45: 129 (11–1,582) 58: 36 (3–419) Other risks factors for cervical cancer after adjusting for HPV: lower education, sexual behavior, and never having a Pap smear	ORs for risk factors other than HPV adjusted for age, and all risk factors considered, including HPV, education, sexual behavior, parity, oral contraceptives, and Pap smear history

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Santos *et al.* 2001 Lima, Peru	Hospital case-control study (1996–1997) Same matched design as Chaouki *et al.* (1998) Cases: 198 women with invasive cervical cancer, 173 with SCC, 15 with ADC and 10 with ADS Controls: 196 women from same hospital	HPV DNA Detected and typed by PCR and Southern blot as described by Chaouki *et al.* (1998) A second step also was performed for 29 cases and 18 controls (randomly chosen) positive for β-globin DNA but negative for the GP5+/6+ primers; DNA was reamplified using E7 primers from high-risk HPV types. 19 of the cases but none of the controls were positive on retesting HPV prevalence 95/18	Age-adjusted ORs for cancer SCC / ADC/ADS 16: 255 (86–759) / 241 (941–1,425) 18: 149 (34–622) / 41 (5–364) 31: 238 (40–1,427) 33: 343 27–4,400) 35: 228 (18–2,926) / 72 (3.2–1,604) 39: 177 (13–2,448) / ∞ 45: ∞ (34–∞) 52: 190 (28–1,289) 58: 67 (8–5,540) Single: 161.9 (59.4–441) / 66.6 (14.1–314) Multiple: 66.6 (14.1–314) / 19.1 (2.1–175) Risk factors for cervical cancer among HPV-positive women were parity, oral contraceptive use, smoking, coca chewing and never using a IUD	ORs for risk factors in HPV-positive strata adjusted for same factors as Chaouki *et al.* (1998) (except residence and family income)
Serology				
Wang *et al.* 1997 Stockholm, Sweden	Cases: 216 women (23–88 years old) with untreated primary invasive cervical carcinoma, incident cases (171 SCC and 45 ADC) Controls: 243 age and sex-matched healthy blood donors (28–80 years old) at three hospitals	HPV serology HPV-16, 18, 33 capsids and E1 and E6 antigens antibodies detected by ELISA HPV seroprevalence 16: 37/18 18: 29/20 33: 31/23	ORs for HPV seropositivity (capsids) 16: 2.7 (1.8–4.2) 18: 1.6 (1.1–2.5) 33: 1.5 (1.0–2.2)	Positive associations also observed with antibodies against some E peptides

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Carter *et al*. 2001 Washington state, USA See Table A-2 for other anogenital cancers	Six case-control studies of anogenital cancer, (1978–1998), part of the CSS cancer registry (SEER, NCI) Cases: 1,782 anogenital cancers including 484 of SCC invasive carcinoma and 305 with ADC and ACIS Controls: 2,383 individuals identified by random-digit dialing matched on age, sex and year of diagnosis	HPV serology (cases and controls) HPV DNA (cases) Same as Madeleine *et al.* 2001 HPV seroprevalence SCC ADC 16 32/15 25/15 18 26/15 39/15 HPV DNA prevalence SCC ADC 16 64 41 18 18 45	Age adjusted ORs for HPV seropositivity and cancer 16 18 SCC 2.7 (2.1–3.5) 2.1 (1.6–2.7) ADC/ACIS 1.8 (1.4–2.5) 3.6 (2.7–4.7) *In situ* 1.7 (1.1.–2.5) 4.1 (2.8–5.8) Invasive 1.9 (1.3–3.0) 2.4 (1.6–3.6)	Stronger association observed for HPV seropositivity when restricted to HPV-DNA-positive cancers

Table A-2. Current nested case-control studies and cohort studies

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
DNA studies					
Longitudinal cohort studies					
Moscicki *et al.* 1998 San Francisco, CA, USA	Longitudinal cohort study on the natural history of HPV infection 618 young women, aged 13–22, who were recently sexually active and found to be HPV positive but without CIN.	33 women developed high-grade SIL, 22 were identified at the first or second visit	HPV DNA Detected by RNA-DNA dot blot hybridization	ORs for High-grade SIL and HPV Low risk + 1 visit 1.0 (0.1–8.0) + 2 visits 3.8 (0.5–32.5) High risk + 1 visit 1.1 (0.2–6.8) + 2 visit 8.9 (1.6–48.3) + ≥3 visits 14.1(2.3–84.5)	Risk for developing SIL was calculated using Cox proportional hazards regression model with time-dependent covariates
Woodman *et al.* 2001 England	Longitudinal cohort study on the natural history of HPV infection 1,075 women (aged 15–19) who visit a clinic between 1988 and 1992 and had recently become sexually active. The women were cytologically normal and HPV negative at recruitment Follow-up – every 6 months Median duration of follow-up 29 months	407 women became HPV positive 246 women developed an abnormal smear 28 women developed high-grade CIN (14 CIN 2 and 14 CIN 3)	HPV DNA Detected by PCR using the GP5+/GP6+ and/or MY09/MY11 primers and typed using type-specific PCR or sequencing primers. Southern analyses also were performed. HPV incidence 38% (407/1,075)	RR for high-grade CIN (CIN II and III) HPV-6 or 11 3.8 (1.5–9.8) HPV-16 8.5 (3.7–19.2) HPV-18 3.3 (1.4–8.1) HPV-31 3.5 (1.0–11.8) HPV-33 0.6 (0.1–4.4) HPV-52 2.3 (0.3–17.2) HPV-58 2.9 (0.8–10.1) Any 7.8 (2.7–22.0)	RRs (relative hazards ratios) for each HPV type were derived from proportional hazards regression models that controlled for other HPV types 5 women who progressed to high-grade CIN consistently tested negative for HPV Limitations: small number of cases and the inclusion of CIN II

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Schlecht *et al.* 2001 Brazil	Ludwig-McGill cohort (1993–1997) Study part of a maternal and child welfare program catering to low-income women in São Paulo 2,528 women aged 18 to 60 were not pregnant, with an intact uterus, were not taking vaginal medication, and had not been treated for cervical disease 1,611 women had valid HPV results for first two visits Follow-up every 4 months the first year and twice yearly thereafter	286 women positive for HPV at enrollment 41 SIL lesions were observed Incidence rate of SIL/1,000 women months 0.73 HPV negative 8.68 HPV positive	HPV DNA Detected by PCR using the MY09/11 primers and typed by hybridization using type-specific probes Oncogenic HPV-16 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 Nononcogenic 6/11, 26, 32, 34, and many others	RR for high-grade SIL (first two vists) One visit only Any HPV 4.1 (1.4–11.7) 16/18 3.9 (0.5–31.1) Two visits Nononcogenic 0 Oncogenic/ not 16 or 18 9.7 (2.6–36.2) 16/18 12.3 (2.6–57.5) Two visits/same type Oncogenic 11.7 (4.1–33.3)	RR modeled by Cox proportional hazard regression Confounders (marital status, education, smoking, feminine hygiene practices, parity, oral contraceptive use, condom use, menopausal status, sexual history) were evaluated for their ability to change an estimate by 10%

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Nested case-control studies					
Liaw *et al.* 1999 Portland OR, USA	Incidence cohort from Kaiser Permanente clinics of 17,654 women 16-years old or older (April 1989 to November 1990) with no previous history or current evidence of SIL/cervical cancer (determined by initial Pap smear); women were followed until December 1994, with some women having intervening voluntary Pap smears	Nested case-control Cases: 380 incident cases (154 ASCUS, 179 LSIL, and 47 HSIL) who were diagnosed at least 9 months subsequent to enrollment and did not have an ambiguous diagnosis at the screening Pap smear Controls: 1,037 (up to 3 per case) matched on age and follow-up time and confirmed as cytologically normal	HPV DNA Detected by PCR using MY09/MY11 primers and typed using type-specific probes High-risk types (HR): 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 Low-risk types (LR): Types other than high-risk HPV prevalence HSIL/control Any 69/16 16 29/3 Other HR 21/6	Adjusted ORs for SIL and HPV HPV at enrollment 16 5.5/5.8/63.9 Other HR 3.5/5.7/30.9 Low risk 4.0/3.2/6.8 HPV at diagnosis 16 14.6/48.4/371.8 Other HR 18.5/90.5/273.9 Low risk 3.8/20.0/33.2 Repeated measurements Highest risk for those positive at both time points ASCUS- atypical squamous cells of undetermined significance SIL- squamous intraepithelial lesions, L- low grade, H-high grade	ORs adjusted for age, enrollment clinic, cytologic diagnosis at enrollment (benign atypia or negative), participation status, follow-up time, number of Pap smears during follow-up DNA testing laboratory changed during study

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Ylitalo *et al.* 1999 Uppsala County, Sweden	Population-based cervical-cancer screening cohort (1969–1995) consisting of women who were born in Sweden, were less than 50 years old, and had a normal Pap smear Follow-up for HPV risk assessment was 0–3 years, using smears taken nearest to diagnosis	Nested case-control Cases: 469 women with carcinoma *in situ* identified by cancer registry Controls: 469 women selected randomly and matched by date of entry into cohort and age and with no history of disease; matched analysis	HPV DNA Detected by PCR assay – multiple smears taken during follow-up HPV prevalence Not reported	Crude OR for HPV and carcinoma *in situ* HPV-16 15.8 (8.0–31.0) HPV-18 2.3 (1.1–5.1) Adjusted ORs for other factors Smoking 1.9 (1.3–2.8) OC 3.6 (1.9–6.9) Risks of OC and smoking similar between HPV-16/18-positive and negative women Number of sexual partners associated with risk in HPV-16/18-negative but not HPV-16/18-positive women	OR for smoking adjusted for years in school, martial status, sexual behavior, age at menarche, parity and OC use OR for OC adjusted for the first five factors listed above and smoking Number of sexual partners not adjusted for HPV types other than 16 and 18
Rozendaal *et al.* 2000 The Netherlands	2,250 women aged 34–54 with no history or present evidence of cervical abnormalities (negative Pap smear) who were part of a screening program (1988–1991) Mean follow-up 6.4 years, with screening every 3 or 5 years.	Entire cohort 13 women with incidence CIN III and 2,237 women without CIN III Nested case-control: Cases: 13 women with CIN III Controls: 640 women selected randomly from the 2,237 women without CIN III	HPV DNA DNA from high-risk types (16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were detected using a PCR-EIA assay HPV prevalence 92/4.9 Cohort 5.4	High-risk HPV Nested case/control OR = 240* Entire cohort (controls 2,237 without CIN III) RR = 210 (27–1,600) * 95% CI not given	

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Coker *et al.* 2001 South Carolina, USA	4,589 women participating in a family-planning program from 11 clinics, mean age 29.3 (1991–1992) - baseline assessment. Low-income, rural and minority population Follow-up: Pap smears during the years 1992–1996	Nested case-control Cases: 426 women with at least one subsequent Pap smear who developed SIL – 22 HSIL and 404 LSIL Controls: 852 (2 per case) matched on Pap smear date and county	HPV DNA Detected using MY09/MY11 primers and typed by hybridization with type-specific probes High risk: 16, 18, 31, 33, 35, 39, 45, 51, 52 Low risk: 6, 11, 42	ORs for HSIL High-risk HPV 3.8 (1.5–9.6) Low-risk HPV 0.4 (0.1–3.2) OR was higher for cases that developed in first year of follow-up (9.1) compared to 4+ years (2.9)	ORs were calculated by logistic regession adjusting for age after evaluating potential confounders Diagnosis was made from Pap smear, which probably resulted in misclassification and a bias towards the null
Wallin *et al.* 1999 Västerbotten county, Sweden	Archival study, population-based cervical-cancer screening cohort consisting of women 25–59 years old (1969–1995) with normal Pap smear and at least one additional smear	Nested case-control Cases: 118 women with invasive cervical cancer (85 with SCC and 19 with ADC, 14 pathological specimens not available) that had been diagnosed after the date of the normal smear Controls: 118 women who did not develop cervical cancer and matched for age, time of the normal Pap smear, and time at which a normal Pap smear was obtained after cancer was diagnosed in the corresponding women.	HPV DNA Nested-PCR using MY09 and MY11 and GP5+ and GP6+ primers. DNA was typed using type-specific PCR HPV prevalence at enrollment 30/2.5	Adjusted OR for invasive cancer HPV 16.4 (4.4–75.1) Persistence* 58.7 (10.2–∞) *Type-specific persistence based on both smears HPV detected: 16 (majority), 18, 31, 33, and 73 Average time to cancer development: 5.6 years (0.5 month to 26.2 years)	OR calculated by conditional logistic-regression analysis with matching for age and the times at which the samples were taken Storage of samples may have led to an underestimation of HPV positivity – only 77% of biopsy specimens were HPV-positive

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Serology					
Chua *et al.* 1996 Västerbotten County, Sweden	Population-based cohort: blood samples from 15,234 women initiated in 1987, matched to morphology registry of cervical smear after a mean follow-up of 34.9 months (range 0.5–76.4) Mean time lag between blood sampling and taking of a smear was 8.7 months, and mean lag-time between sampling and diagnosis of CIN was 13.3 months	Nested case-control Cases: 74 women with CIN; 10 CIN I, 41 high-grade CIN II or III, and 23 CIN-NOS (undeterminable) identified by morphology registry and confirmed by two reviewers Controls: 142 women; 2 per case (with 6 exceptions lacking stored Pap smears) with no CIN diagnosis during follow-up and matched for age, date of blood sampling, and residence	HPV serology Capsid antibodies to HPV-16, 18, and 33 HPV seroprevalence 16 36/16 18 18/15 33 20/18 HPV DNA Detected from cervical smears by nested-PCR assay using MY09/MY11 and GP5+/GP6+ primers and typed using type-specific primers (6, 16, and 18) HPV DNA prevalence 16 44/1 All 88/4	Adjusted ORs for HPV seropositivity and CIN development 16 3.0 (1.5–6.0) 18 1.2 (0.5–2.7) 33 1.1 (0.5–2.4) Adjusted ORs for CIN II and III and HPV-16 Seropositivity 2.8 (1.1–7.2) DNA 71.3 (10.3–3,113) Agreement between HPV-16 PCR and HPV-16 serology (kappa (95% CI)) was 0.52 (0.26–0.77) for difference in sampling less than 6 months and 0.25 (0.08–0.42) for sampling greater than 6 months apart	ORs calculated by conditional logistic regression of matched samples HPV DNA estimates are not prospective; exposure assessed at time of diagnosis

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Vonka *et al.* 1999 Prague	More than 10,000 women between 25 and 45 (1975–1983) with no history of treatment for cervical neoplasia; all women at enrollment were examined cytologically and colposcopically, donated a blood sample, and answered a detailed questionnaire Subjects free of pathological findings were included in study and followed at 2-year intervals	Nested case control Cases: (Group II) 67 women who developed cervical neoplasia (ranging from moderate dysplasia to invasive carcinoma), second serum sample available on subset of 19 cases Cases: (Group I – not prospective) 115 women diagnosed with cervical neoplasia (ranging from moderate dysplasia to invasive carcinoma) at enrollment Controls: 129 healthy women who did not develop pathology (1–3 per case) matched by age, sexual behavior, smoking habits and DKG (diathermo-electrocoagulation of the ectopic epithelium and transformation zone)	HPV serology HPV-16, 18, and 33 VLP (capsid antibodies) and nonstructural (E) antibodies HPV seroprevalence (VLP) Group I 32/14 Group II 25/14	ORs for capsid antibodies Group I HPV-16 2.7 (1.2–5.9) HPV-18 3.4 (1.6–7.5) HPV-33 2.7 (1.2–5.9) Any HPV 2.9 (1.5–5.3) Group II HPV-16 3.9 (1.1–13.9) HPV-18 2.7 (0.9–8.6) HPV-33 1.5 (0.6–4.1) Any HPV 2.1 (0.9–4.7) ORs for nonstructural antibodies (E2, E4, E7) Risks only significant for Group I Seroconversion occurred in 58% of patients	Cases of CIN (ranging from moderate dysplasia to invasive carcinoma) were combined because no difference was observed in antibody titers among diagnoses Group II contained 34 women with suspicious colposcopical or cytological findings, which were probably of inflammatory nature at enrollment

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Lehtinen *et al.* 1996 Finland	Finnish Social Insurance Institution (1966–1972) cohort consisting of 18,814 women free of cancer and who had donated blood; followed for 23 years	Nested case-control Cases: 72 (27 with invasive carcinoma and 45 with *in situ* carcinoma) women identified by cancer registry Controls: 143 women matched for age and municipality	HPV-16 serology Capsid antibodies Other exposures: *Chlamydia*, and Herpes simplex 2 (HSV-2) serology HPV-16 seroprevalence 24/2	Adjusted OR for *in situ* and invasive cervical cancer HPV-16 12.5 (2.7–57)* HSV 1.5 (0.4–5.8) *Chlamydia* 1.8 (0.9–3.4) *Higher risk observed with longer lag time (between serum sample and diagnosis)	ORs (HPV) adjusted for smoking and STD ORs (STD) adjusted for smoking

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Dillner *et al*. 1997 Finland, Norway, and Sweden	Three population-based serum bank cohorts followed until 1992: 1. Finnish maternity cohort (1983–present): 710,000 blood samples from 390,000 women collected at maternity clinics 2. JANUS (Norway) Phase I (1974–1978) Phase II (40–42 yrs 1983–1988), combined 425,000 blood samples from 294,000 women 3. Västerbotten project (Sweden) (1986–present) blood samples from > 15,000 women	Nested case-control Cases: 182 cervical carcinoma diagnosed after enrollment in cohort and identified by cancer registries Controls: 538 women (3 per case) free of cervical cancer for equal length of follow-up and matched for length of serum storage and age (8 serum samples could not be located)	HPV serology Capsid antibodies Other exposures *Chlamydia* serology HPV seroprevalence 16 16/7 18 14/8 33 17/11	Adjusted RR for cervical cancer all cohorts combined HPV-16 2.2 (1.2-3.9) HPV-18 1.8 (1.0-3.3) HPV-33 1.5 (0.9-2.6) SCC HPV-16 3.2 (1.7–6.2) HPV-18 1.5 (0.8–2.9) HPV-33 1.6 (0.9–2.7) ADC HPV-16 Not estimated HPV-18 3.4 (0.8–14.9) HPV-33 1.7 (0.3–8.5) Increase in risk with increasing lag time; no increase in risk with increasing antibody levels Smoking 1.5 (1.0–2.1) *Chlamydia* 1.8 (1.2–2.9) RR for HPV-16 stratified by STD High STD, 1.1; low STD, 9.3	RR (HPV) adjusted for *Chlamydia* and smoking, RR (smoking) adjusted for *Chlamydia* and HPV-16 seropositivity RR (*Chlamydia*) adjusted for HPV-16 seropositivity and smoking

Reference	Cohort	Cases and controls or study population	Exposure: detection method and prevalence (%)	Effects: RR or OR (95% CI)	Comments
Shah *et al.* 1997 Washington County, Maryland, USA	Population-based cohort (1974–1975): blood and brief questionnaire collected from 11,009 women older than 18 years and followed until 1990	Nested case-control Cases: 42 cases of *in situ* (28) or invasive (14) cervical cancer with serum specimen Controls: 83 (2 per case except one case) not in cancer registry and alive at the time the case was diagnosed; matched for age, race, date of blood collection, hours since last meal, and time since last menstrual period	HPV serology HPV-16 and HPV-6 VLP seropositivity was measured by an ELISA assay measuring amount of absorbance HPV seroprevalence 16 ≥ 200 33/12 16 ≥ 400 19/4 6 ≥ 600 33/25	Adjusted OR for *in situ* and invasive cervical cancer HPV-16 absorbance ≥ 200 3.9 (1.4–10.7) ≥ 400 7.5 (1.5–36.3) HPV-6 absorbance ≥ 600 1.4 (0.7–3.1)	ORs adjusted for matched variables: age, race, date of blood collection, hours since last meal, and time since last menstrual period

Table A-3. Current longitudinal studies: CIN persistence or progression

Reference	Cohort and follow-up	Study population at the end of follow-up and for analyses	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Remmink *et al.* 1995 Amsterdam, the Netherlands	342 outpatients aged 16–55 identified from 1990–1992 with abnormal cervical cytology without prior cervical pathology and no concomitant cancer or history of prenatal DES exposure Follow-up: every 3–4 months by cytology and HPV typing, mean duration of follow-up 16.5 months; biopsy taken on women developing progressive CIN disease	CIN progression at end of study 19 patients progressed to CIN III HPV patient groups (status during study) used to calculate hazard rate High risk 202 Undefined 41 Low risk/ Negative 99	HPV DNA Detected by PCR using GP5/GP6 primers and genotyped by type-specific PCR and/or hybridization with type-specific probes	Adjusted OR for high-risk HPV and progressive CIN 6.0 (1.3–27.4) CIN progression related to HPV type High-risk HPV compared to other HPV groups (low risk and undefined) Cox's proportional hazard rate: 6.5 (1.5–28.4); $P = 0.017$	17 of the 19 women with progressive disease had high-risk HPV, and 2 had unidentified HPV subtype OR adjusted for smoking, age, sexarche, number of sexual partners

Reference	Cohort and follow-up	Study population at the end of follow-up and for analyses	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Nobbenhuis *et al.* 1999 Amsterdam, the Netherlands	Colposcopy clinic population (1990–1992); later publication of follow-up of most of the same population (cohort) reported by Remmink above but with 353 women, aged 18–55 Follow-up: every 3 or 4 months by cytology and HPV typing, median follow-up 33 months, median no. of visits 9, biopsy taken (for most patients) at last visit	CIN progression at end of study 33 women with clinical progression and 103 with CIN III HPV status at baseline and during study 233 women had high-risk HPV at baseline; 122 of whom had persistent infection 120 women were HPV negative at baseline, 39 acquired high-risk infection	HPV DNA Detected by PCR-EIA High risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 tested in one assay HPV prevalence at baseline Cohort – 66%	Clinical progression/HPV infection All 33 had persistent high-risk HPV ORs for CIN III High-risk HPV DNA Baseline 29 (9.2–96) Persistent 327 (42–2,468) Clearance/ acquisition 2.9 (0.2–20) Median clearance of high-risk HPV HPV at baseline 25 months New acquisition 6 months Persistent infection Positive HPV at baseline till last smear Clearance/acquisition Acquired or cleared HPV infection or both	No additional risk for CIN III (end histology) found for age, smoking, or sexual behavior; clinical progression (defined as covering 3 or more cervical quadrants), or suspected microinvasive carcinoma

Reference	Cohort and follow-up	Study population at the end of follow-up and for analyses	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Cavalcanti *et al.* 2000 Rio de Janeiro, Brazil	Series of 514 cases of normal, benign, premalignant, and malignant lesions (1990–1997) from hospitals and clinics Follow-up: 8 years, with average interval of 6 months	Retrospective study 280 untreated patients included in analysis Cases: 82 with clinical progression to carcinoma *in situ* or invasive cancer Controls: 76 with lesions that regressed spontaneously 122 women had persistent lesions	HPV DNA Detected by *in situ* hybridization HPV prevalence* Cases/"controls" 6/11 2/64 16/18 80/29 31/33/35 18/20 *Includes multiple infections	Adjusted ORs progression of SIL to cancer HPV types 6/11 0.02 (0.0–0.2) 16/18 13.2 (1.9–58.6) 31/33/35 1.2 (0.2–7.3) Other factors STD 13.0 (1.9–56.3) No significant risk associated with tobacco or oral contraceptive use	ORs adjusted in multivariate analysis for the other risk factors, HPV, STD, tobacco, and oral contraceptives

Table A-4. Current human studies of noncervical anogenital cancer

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Bjørge *et al.* 1997 Norway	Multiple	Prospective Cohorts: two serum banks, Finnish maternity cohort (390,000 women) and The Janus project (300,000) in Norway, mean follow-up 8 years Cases: 81 (19 from Finland and 62 from Norway) patients with noncervical anogenital cancer identified from cancer registry Controls: 240 (56 from Finland, 184 from Norway) matched (~3 per case) for age, age at serum sampling, storage time, and county (in Norway)	HPV serology Capsid antibodies for HPV-16, 18, 33 Preassigned cut off point HPV-16 = 0.239 HPV-18, 33 = 0.100 Similar results if 0.200 was used HPV-16, 18, 33 seroprevalence (all cases) 35/21	ORs (> 1) for all anogenital 16 3.1 (1.4–6.9)$^{+}$ 18 1.2 (0.4–3.0) 33 2.8 (1.0–8.3)$^{++}$ Vulvar/vaginal 16 4.5 (1.1–22)$^{++}$ 18 1.5 (0.3–7.5) 33 3.3 (0.5–23) Preinvasive vulvar/vaginal 16 ∞ (3.8–∞) 18 12 (1.2–590) 33 4.5 (0.5–54) *Chlamydia* ∞ (0.5–∞) Penile/scrotal 16 7.2 (0.5–390) 18 0.7 (0.0–7.6) 33 7.2 (0.5–390) *Chlamydia* 2.3 (0.5–10) Anal 16 1.5 (0.3–5.6) 18 1.5 (0.2–9.6) 33 0.8 (0.1–6.2)	Only ORs > 1 are reported in this table $^{+}$ORs still significant after adjusting for HPV-33 $^{++}$ORs still elevated but no longer significant after adjusting for HPV-16 or 33 ORs for *Chlamydia* only given when > 1.0

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Strickler *et al.* 1998 Minnesota	Multiple	Cross-sectional study using NCI Immunodiagnosis Serum Bank established in collaboration with Mayo Clinic (1975–1991) Cases: 905 hospitalized patients with 21 selected cancers related to HPV-16 Controls: 48 noncancer patients (endocrine disease)	HPV serology HPV-16 VLP HPV seroprevalence Controls 4 Penis 63 Ovary 4 Uterus 9 Vagina 27 Vulva 27 Cervix 52 Aerodigestive most < 10%		Small sample size for each specific cancer (approximately 50) and controls; some cancers, very small sample size, (fewer than 20 subjects for vagina and penis); mean age varied by cancer site, prevalence

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Carter *et al.* 2001 Washington State, USA	Multiple (including cervical, see Carter *et al.* 2001, in Table A-1 (Appendix A))	Population-based case-control studies for noncervical anogenital cancer – see Table A-1 (Appendix A) Cases: vulvar – 535; vaginal – 140; anal (female) – 109; anal (male) – 88; and penile – 121 Controls: 2,388 individuals identified by random-digit dialing matched on age, sex, and year of diagnosis	HPV serology and DNA See Table A-1 (Appendix A) HPV-16 seroprevalence Vulvar 43/15 Vaginal 44/15 Anal-female 42/15 Anal-male 52/16 Penile 24/13 DNA prevalence (cases) Vulvar 89 Vaginal 91 Anal 94 Penile 82	Age-adjusted ORs for HPV seropositivity and cancer (*in situ* plus invasive) HPV-16 seropositivity Vulvar 4.5 (3.8–6.1) Vaginal 4.8 (3.3–6.9) Anal (female) 4.4 (2.9–6.7) Anal (male) 5.9 (3.4–10.3) Penile 2.2 (1.4–3.6) HPV-18 seropositivity Vulvar 2.0 (1.6–2.6) Vaginal 2.2 (1.5–3.2) Anal (female) 2.7 (1.7–4.1)	Stronger association observed for HPV seropositivity when restricted to HPV-DNA-positive cancers

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Hildesheim *et al.* 1997a Chicago and upstate New York	Vaginal	Population-based case-control study (1985–1987); 12 months retrospective and 18 months prospective ascertainment Cases: 23 confirmed *in situ* or invasive vaginal cancer who agreed to interview and donated a blood sample Controls: 28 controls identified by random-digit dialing and matched on age, race, and residence who agreed to an interview and donated a blood sample	HPV serology HPV-16 VLP Seropositivity cutoff OD = 1.0 and high titer OD ≥ 1.5 normalized to control sera HPV-16 seroprevalence 50/25	Age-adjusted RRs for *in situ* and invasive vaginal cancer HPV-16 3.5 (1.0–13) High titer 33 (2.5–430) HSV-2 3.0 (0.6–15) *Chlamydia* 4.6 (1.2–18)	Adjusting RR for HSV-2 and *Chlamydia* did not affect OR for HPV Limitations: very small sample size, vaginal cancer is very rare

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Sun *et al.* 1996 Chicago and New York, USA	Vulvar	Subset of multicenter case-control study (Brinton *et al.* 1990) of vulvar carcinoma Cases: 54 women with vulvar neoplasia: 14 with BWSCC (basaloid or warty carcinomas), 22 with VIN (vulvar intraepithelial neoplasia) and 18 with KSCC (keratinizing SCC of the vulva) Controls: 44 women with serological specimens originally identified by random-digit dialing	HPV serology HPV-16 VLP antibodies and HPV E6 and E7 HPV-16 VLP seroprevalence KSCC 22 VIN 59 BWSCC 50 Controls 18	Age-adjusted ORs for vulvar neoplasia HPV -16 VLP KSCC 1.3 (0.3–4.9) VIN 5.4 (1.7–18) BWSCC 4.5 (1.2–16) HPV 16 E6 KSCC 1.1 (0.1–13) VIN 1.3 (0.1–16) BWSCC 3.9 (0.5–32) HPV 16 E6 or E7 KSCC 1.1 (0.1–13) VIN 2.8 (0.4–20) BWSCC 13.8 (2.1–89)	ORs could not be calculated for E7 antibodies since no controls had antibodies to E7 KSCC is found in older women, does not have adjacent VIN, and is not strongly related to sexual history or HPV sequences Limitations: Only a small portion of subjects (with available sample) from original population was examined for this report

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Hildesheim *et al.* 1997b Upstate New York and Chicago, USA	Vulvar	Population from Brinton *et al.* (1990) (1985–1987), may include cases from Sun *et al.* (1996) (above); 12 months of retrospective and 18 months of prospective case ascertainment Cases: 142 women with VIN III or invasive carcinoma (107 from New York and 35 from Chicago) with blood specimens and interviews Controls: 126 women (121 from New York and 5 from Chicago) identified by random-digit dialing and matched by age, race, and residence with blood specimens and interviews	HPV serology HPV-16 capsid antibodies HPV-16 seroprevalence 44/12 Other infectious diseases HSV-1, HSV-2, and *Chlamydia* serology	Adjusted ORs for HPV-16 seropositivity All cases 5.3 (2.5–11.1) All cases/ High titer 20.1 (5.4–76.7) VIN III 13.4 (3.9–46.5) All invasive 2.9 (0.9–8.7) BWSCC 3.8 (0.8–18.9) KSCC 1.6 (0.4–7.4) Other risk factors: significant associations observed for number of lifetime sexual partners, HSV-1, HSV-2 after adjusting for HPV Age-adjusted ORs for HPV-16 stratified by other risk factors HSV Negative 14.7 (1.8–120) HSV-1 only 6.7 (2.7–17) HSV-2 2.1 (0.5–9.0) Cigarette smoking Never 3.4 (0.9–13) Ever 8.5 (3.8–19) No heterogeneity was observed for HPV within strata by number of sexual partners, oral contraceptive use, or *Chlamydia*	ORs for HPV adjusted for HSV, *Chlamydia*, age, number of sexual partners, education, age started cigarette smoking, and oral contraceptives ORs for other risk factors adjusted for above and HPV Limitations: Only assayed for HPV-16 Low response rate to donate blood may have led to a differential response in cases and controls with respect to age, smoking, and race, which may have biased estimates

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Madeleine *et al.* 1997 Washington State, USA	Vulvar	Population case-control study (1980–1994) Cases: 510 incident cases (400 *in situ* and 110 with invasive disease) identified by Cancer Surveillance Registry System Controls: 1,403 women identified by random-digit dialing and frequency matched by age with no history of vulvar cancer (may be one of six case-control studies reported by Carter *et al.* 1997, see above)	HPV serology HPV-6, 16, 18 capsid antibodies in cases and controls HPV seroprevalence Invasive/*in situ*/control 16 44/53/22 18 11/13/10 6 48/50/36	Adjusted ORs for *in situ* HPV-16 3.6 (2.6–4.8) HPV-6 1.2 (0.8–1.7) HPV-18 0.9 (0.6–1.5) Smoking 4.4 (3.1–6.2) HSV-2 1.9 (1.4–2.6) Adjusted ORs for invasive cancer HPV-16 2.8 (1.7–4.7) HPV-6 1.2 (0.7–2.3) HPV-18 1.2 (0.5–2.7) Smoking 2.2 (1.3–3.7) HSV-2 1.5 (0.9–2.6) Age-adjusted ORs for vulvar cancer from smoking and HPV-16 seropositivity Smoking/antibody status Never/negative 1.0 (ref) Never/positive 2.9 (1.7–5.0) Current/negative 4.9 (3.3–7.5) Current/positive 18.8 (12–30) Dose/response relationship observed with smoking	ORs for *in situ* adjusted for age, education, smoking, and HPV-16 serology ORs for invasive adjusted for above plus body mass, smoking and HPV-16; test for interaction was not significant (0.05 level) on the multiplicative scale but was on the additive scale ($P < 0.001$) Limitations: Low participation level (70%) All histological types included

Reference	Cancer type	Study design and population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI) or other measures	Comments, adjustments, limitations
Wideroff *et al.* 1996 Hunan Province, China	Penile	Population-based study, cases and controls prospectively ascertained Cases: 55 cases with newly diagnosed penile cancer Controls: 60 community controls matched for age and residence	HPV serology HPV-16 capsid antibodies measured from frozen serum samples using a cut of OD = 0.72	Median OD values (HPV-16) Cases 0.39 Controls 0.36	
Frisch *et al.* 1997 Denmark and Sweden	Anal	Subset of a population-based case-control study (1991–1994) Cases: 388 of original 417 patients with invasive or *in situ* cancer Cancer controls: 20 cancer controls from the original study having 534 cancer controls (with ADC of the rectum) matched for age, sex, county, and year of diagnosis and 554 population controls from national population registers	HPV DNA from tissue Detected by PCR using GP5+/6+ primers and typed by specific probes HPV DNA prevalence Women / Men HR 89 / 69 16 77 / 57 LR 4 / 6 All 20 cancer controls were negative High risk (HR): 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 Low risk (LR): 6, 11, 40, 42, 43, and 44	Anal cancer was related in both men and women to measures of sexual promiscuity including anal warts	Limitations: HPV DNA measured in 388 patients with anal cancer and only 20 cancer controls and not measured in population controls

Table A-5. Current human studies on head- and neck-related cancers

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Nishioka *et al.* 1999 Japan	Head and neck	Hospital case-control study (1990–1993) Cases: 74 patients with head and neck cancer Controls: 70 hospital controls with benign lesions matched on anatomical site, age, sex, and cigarette smoking	HPV-16/18 DNA Detected from surgical tissue by PCR and slot-blot hybridization HPV/DNA prevalence 16/4	Crude ORs for head and neck cancer HPV 4.3 (1.3–14.8) All cases and controls were HPV-16 except one case, which was 16 and 18	Limitations: small sample size, crude OR

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Gillison *et al.* 2000 Maryland	Head and neck	Case-case study, 253 patients with newly diagnosed or recurrent head and neck SCCs, 62 HPV-positive and 191 HPV-negative patients Survival study Available for 252 patients, median follow-up of 31 months, median overall survival 85 months	HPV DNA Detected from tumors by PCR using MY09/MY11 primers and E7 primers, Southern blot (HPV-16 positive), sequencing and *in situ* hybridization also done HPV prevalence Overall 25 All but one high risk (16, 18, 31, or 33)	Multivariate analysis for HPV positivity Oropharyngeal 6.2 (3.1–12.1) Poor tumor grade 2.4 (1.2–4.9) Univariate analysis (OR) for HPV stratified by oropharyngeal (OP) and nonoropharyngeal tumor site (NOP) OP / NOP Tobacco 0.16 / 2.0 Alcohol 0.17 / 1.7 Basaloid 18.7 / 8.9 Mutant p53 0.06 / 1.6 Survival analysis: adjusted hazard ratio (HR) for HPV positivity (reference HPV negative) All deaths 0.6 (0.4–1.0) Only cancer deaths 0.4 (0.2–0.9)	Southern blot analysis: consistent with viral integration Multivariate analysis: ORs adjusted for sex, age, race, tobacco, alcohol, primary tumor, lymph node status, tumor grade, basaloid morphology, p53 mutations, and primary therapy Survival analysis: adjusted for lymph node status, age, and alcohol consumption

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Mork *et al.* 2001 Norway, Finland, and Sweden	Head and neck	Prospective/nested case-control study Cohort: four serum banks from Norway, Finland and Sweden Cases: 292 patients with squamous-cell carcinoma of the head and neck identified (1997) from cancer registries Controls: 1,568 matched to cases for sex, age at diagnosis, and length of serum storage, 5 (Norway and Sweden) or 7 (Finland) per case	HPV serology HPV-16, 18, 33, 73 capsid antibodies HPV DNA Detected from biopsies (cases only) by PCR using GP5+/GP6+ (L1) and CpI/CpIIG (E1) primers	Adjusted ORs for head and neck cancer and HPV seropositivity HPV-16 2.2 (1.4–3.4) HPV-18 1.0 (0.6–1.8) HPV-33 0.8 (0.5–1.3) HPV-73 0.6 (0.4–1.2) Adjusted OR for HPV-16 seropositivity and epithelial type Skin 0.5 (0.1–2.1) Respiratory 2.8 (0.5–15.9) Mucosal stratified squamous 2.6 (1.7–4.2) Tumor type and HPV-16 seropositivity Significantly elevated ORs observed for tongue (2.8), oropharynx (14.4), and larynx (2.4)	ORs adjusted for cotinine levels; no adjustment for alcohol use

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Schwartz *et al.* 1998 Washington State, USA	Oral	Population case-control study (1990–1995) Cases: 284 cases with SCC of the oral cavity (261 with invasive and 23 with *in situ*) identified by the Cancer Surveillance System, part of SEER Controls: 477 sex- and age-matched controls (3/2 cases) identified by random-digit dialing	HPV DNA Detected from exfoliated oral tissue (cases and controls) or tumors (cases) by PCR using MY09/11 primers and typed using specific probes HPV High-Risk DNA prevalence 5.9/4.1 HPV serology HPV-16 capsids antibodies HPV-16 seroprevalence 51/35	Adjusted ORs for oral cancer (exfoliated oral tissue) HPV DNA Any 0.9 (0.5–1.6) 6 or 11 0.5 (0.2–1.40) 16, 18, 31/33/35 1.3 (0.6–2.9) HPV-16 seropositivity Total 2.3 (1.6–3.3) HPV-16 DNA 6.8 (3.0–15.2) HPV-6/11 DNA 1.2 (0.4–3.8) Smoking and HPV-16 Antibody/current smoking Negative/yes 3.2 (2.0–5.2) Positive/no 1.7 (1.1–2.6) Positive/yes 8.5 (5.1–14.4) Synergy index 2.6 (1.3–5.0)	ORs adjusted for age, sex, smoking, and alcohol ORs for joint association adjusted for age, sex, and alcohol Limitations: DNA isolated from exfoliated tissue may not have been a good measure of cumulative effects of past infection Low participation rate

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Smith *et al.* 1998 Iowa, USA	Oral	Hospital case-control study (1994–1996) Cases: 93 newly diagnosed oral or pharyngeal cancer Controls: 205 age- and gender-matched with no history of oral cancer	HPV DNA Detected from exfoliated squamous cells (mouth rinse) by PCR using MY09/MY11 primers and identified by probes and sequencing HPV prevalence All 15/5 16 43/20	Adjusted ORs for oral cancer HPV 3.7 (1.5–9.3)	ORs adjusted for age, tobacco and alcohol use; other potential factors did not alter risk and were not included in the analysis
Mellin *et al.* 2000 Sweden	Tonsillar	Longitudinal study (survival) (1984–1996) Cases: 60 hospital patients with tonsillar SCC undergoing treatment and with good quality DNA Controls: 10 patients who had undergone tonsillectomy with available tonsillar tissue	HPV DNA Detected from tissue by PCR using GP5+/6+ primers and typed by specific primers HPV DNA prevalence 43/0	HPV types HPV-16 was found in all cases, one case had 16 and 33 HPV-16 and tumor characteristics and prognosis HPV-16 detection was significantly related to stage (less advanced), and size (smaller), and prognosis (lower risk of relapse and improved survival), but not to lymph node metastases or histology	Prognosis for tonsillar cancer plus HPV was independent of stage, nodal status, gender, or age

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Dillner *et al.* 1995 Finland	Esophageal	Prospective study (1968–1972) Social Insurance Institution cohort-serum samples Cases: 165 head and neck, 39 esophageal (29 with SCC) as of 1991 Controls: 330 (2 per case) individuals free of cancer, matched for sex, age, and municipality	HPV serology HPV-16 capsid antibodies, using 0.180 absorbance units as a cut off point HPV-16 seroprevalence esophageal cancer 21/3	Adjusted ORs for cancer site and HPV-16 seropositivity Esophagus 13.1 (1.6–108) Larynx 0.2 (0.0–2.0) Lip, tongue, Salivary 0.6 (0.2–2.1) Other oral 0.4 (0.0–7.1) No difference in percent of HPV-16 seropositivity between all cases and controls	ORs adjusted for smoking Limitations: Stratification by tumor site resulted in fewer than 100 cases/site, small number of exposed cases
Han *et al.* 1996 Shaanzi Province, China	Esophageal	Hospital case-control study Cases: 90 esophageal (1995) from two cancer hospitals, most (95%) SCC Controls: 121 noncancer patients (routine exams or disorders unrelated to HPV), matched for sex and age	HPV serology (virus like particles) HPV-16 VLP measured by ELISA; BPV (bovine papillomavirus) VLP also measured HPV-16 seroprevalence (cutoff OD = 1.0) 24/7	Mean ELISA OD HPV / BPV Cases 0.85^{+} / 0.81^{++} Controls 0.74^{+} / 0.88^{++} $^{+}P < 0.001$; $^{++}P = 0.12$ ORs for HPV-16 seropositivity cutoff OD 0.7 2.2 (1.2–4.3) 1.0 4.5 (1.8–11.9) 1.1 6.3 (1.6–23.7)	OD for ELISA normalized to optical density of serum pool ORs adjusted for sex and age

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Bjørge *et al.* 1997 Norway	Esophageal	Prospective study (1973–1997) Janus serum bank cohort (500,000 donors) Nested case-control Cases: 57 cases of esophageal cancer identified from cancer registry Controls: 171 (3 controls per case) individually matched for sex, age, serum sampling and storage time, and county	HPV serology HPV-16, 18, 33 capsids antibodies measured by ELISA using two absorbance cutoffs (0.100 and 0.200) except for HPV-16 (0.100 and 0.239) HPV-16, 18, and 33 prevalence 21/11	Adjusted ORs for esophageal cancer HPV-16 0.100 2.9 (0.8–10) 0.239 6.2 (1.0–67) HPV-18 0.100 2.2 (0.7–6.7) 0.200 2.3 (0.6–7.6) HPV-33 0.100 2.2 (0.7–7.3) 0.200 4.5 (1.1–21) 16/18/33 2.2 (0.9–5.3) ORs (not adjusted) for SCC cases HPV-18 and 33 elevated but not significant HPV-16 0.239 10.0 (1.0–510)	ORs adjusted for cotinine (smoking) Combined 16/18/33, cutoffs: HPV-16 – 0.239; HPV 18 and 33 – 0.1

Reference	Cancer Type	Study design and population	Measurement of exposure and HPV prevalence (population or cases/controls)	Effects: RR or OR (95% CI)	Comments
Lagergren *et al.* 1999 Sweden	Esophageal	Population case-control study (1994–1997) Cases: 173 incident ADC of the esophagus or gastroesphageal junction and 121 incident SCC from tumor registries and surgery/pathology departments Controls: 302 controls (CON) randomly selected from population register	HPV serology HPV-16 and 18 capsid antibodies by ELISA using 0.100 and 0.261 absorbance units as cut-off values HPV seroprevalence SCC/ADC/CON 16 12/12/11 18 5/2/10	Adjusted ORs for SCC (> 0.100 absorbance units) HPV-16 0.9 (0.3–2.4) HPV-18 0.3 (0.1–1.2) Adjusted ORs for ADC (> 0.100 absorbance units) HPV-16 1.4 (0.7–2.6) HPV-18 0.2 (0.1–0.7)	ORs adjusted for age, sex, smoking, status, alcohol and education Multivariate analysis restricted to 62 SCC, 155 ADC patients, and 302 controls No associations were observed in the age- and sex-adjusted analyses using either 0.100 or 0.261 values

Table A-6. Current cohort studies on HPV viral persistence

Reference	Cohort and follow-up	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Ho *et al.* 1995 Bronx, NY, USA	Cohort: 70 women with CIN II, no CIN treatment within the past 6 months (1991–1994) Follow-up: colposcopy and Pap smear every 6 weeks or 3 months for 15 months, biopsy at 9 months, total of 532 visits, median 6 visits/subject (range 1 to 7)	Population at the end of the study SIL status end of study 40 clear (+/-) 186 persistent (+/+) Based on 226 pairs of observation with known SIL status at consecutive visits t and t +1 (data from individuals with normal smears were excluded – 30 women of original 100)	HPV DNA Detected by PCR and Southern blot analysis High-risk (HR): 16, 18, 31, 33, 35, 39, 45, 51, 52, 58	ORs for persistent SIL (reference – HPV not detected at both visits) Southern: persistent HPV (same or different types found at t and t+1 vists) Same 2.6 (1.0–6.6) Different 4.5 (1.4–14.3) PCR: persistent HPV Same 3.8 (1.3–11.2) Different 1.9 (0.6–6.6)	ORs adjusted for randomized group and repeated measurements Persistent SIL or HPV defined as continual occurrence of SIL or HPV in two consecutive visits Other factors such as age, Hispanic ethnicity, education, smoking, oral contraceptives, and sexual behavior did not correlate with the risk of persistent SIL Study originally designed as randomized, double-blinded placebo-controlled clinical trial for evaluating the efficacy of using beta carotene for the treatment of CIN

Reference	Cohort and follow-up	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Moscicki *et al.* 1998 (also discussed in Table 3.4)	Longitudinal cohort study on the natural history of HPV infection 618 young women, aged 13–22, who were recently sexually active and found to be HPV positive but without CIN	33 women developed high-grade SIL, 22 were identified at the first or second visit	HPV DNA Detected by RNA-DNA dot blot hybridization	ORs for High-grade SIL and HPV Low-risk HPV + 1 visit 1.0 (0.1–8.0) + 2 visits 3.8 (0.5–32.5) High-risk HPV + 1 visit 1.1 (0.2–6.8) + 2 visit 8.9 (1.6–48.3) + ≥3 visits 14.1 (2.3–84.5)	Risk for developing SIL was calculated using Cox proportional hazards regression model with time-dependent covariates
Ylitalo *et al.* 2000a Uppsala County, Sweden	Same cohort as Ylitalo *et al.* (1999) with normal Pap smear with a median follow-up of 8 years (< 1 to 25 years)	Cases: 484 women with carcinoma *in situ* identified by cancer registry Controls: 619 women selected at random and matched by date of entry into cohort, age, and with no history of disease matched analysis	HPV-16 DNA Detected by real-time PCR method in multiple samples taken throughout study HPV prevalence (first smear) 26.9/5.8	Crude OR for carcinoma *in situ* Last two smears Neg.-neg. 1.0 (ref) Pos.-neg. 4.9 (2.1–11.3) Neg.-pos. 9.7 (4.3–21.8) Pos.-pos. 31.2 (10.6–91.8) Probability of HPV-16 positivity among cases increased with increasing years before diagnosis Estimated median incubation period for women diagnosed ≥ 35 years 7–12 years < 35 years 5–8 years	RR modeled by Cox Proportional hazard regression Confounders (marital status, education, smoking, feminine hygiene practices, parity, oral contraceptive use, condom use, menopausal status, sexual history) were evaluated for their ability to change an estimate by 10%

Reference	Cohort and follow-up	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Schlecht *et al.* 2001 Brazil also discussed in Table 3.4	Ludwig-McGill cohort (1993–1997) Study part of a maternal and child welfare program catering to low-income women in São Paulo 2,528 women between the ages of 18 to 60 who were not pregnant, with an intact uterus, were not taking vaginal medication, and had not been treated for cervical disease 1,611 women had valid HPV results for first two visits Follow-up every 4 months the first year and twice yearly thereafter	286 women positive for HPV at enrollment 41 SIL lesions were observed Incidence rate of SIL/1,000 women months 0.73 HPV negative 8.68 HPV positive	HPV DNA Detected by PCR using the MY09/11 primers and typed by hybridization using type-specific probes Oncogenic HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 Nononcogenic 6/11, 26, 32, 34, and many others	RR for high-grade SIL (first two vists) One visit only Any HPV 4.1 (1.4–11.7) 16/18 3.9 (0.5–31.1) Two visits Nononcogenic 0 Oncogenic/ not 16 or 18 9.7 (2.6–36.2) 16/18 12.3 (2.6–57.5) Two visits/same type Oncogenic 11.7 (4.1–33.3)	RR modeled by Cox proportional hazard regression Confounders (marital status, education, smoking, feminine hygiene practices, parity, oral contraceptive use, condom use, menopausal status, sexual history) were evaluated for their ability to change an estimate by 10%

Table A-7. Current case-control and cohort studies on HPV variants

Reference	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Case-control studies				
Song *et al.* 1997 Seoul, Korea	Cases: 70 women with CIN (21 CIN III and 49 cervical carcinoma) Controls: 87 patients with no cervical pathology	HPV DNA Detected by PCR using consensus primers for 16, 18 and 33 and HPV-16 variant identified by PCR-directed sequencing HPV-16 prevalence Cancer/CIN/controls 55/29/10	Percent of HPV-16 E7 variants KE7-1 / others[a] Control 33 / 67 CIN 3 50 / 50+ Invasive 70 / 30++ [a] HPV-16 prototype and other variants with no amino acid change Test for trend: Variant according to pathologic grade tested by likelihood ratio +P = 0.043 ++P = 0.0001	KE7-1 variant (nucleotide 647 A to G), most common and only variant detected associated with an amino acid change (Asp to Ser at codon 29)
Bible *et al.* 2000 London, England	89 women positive for HPV-16 E5 DNA, attending outpatient clinics or women's centers 25 normal cytology/ border line lesions 18 CIN I 17 CIN II 19 CIN III 4 cervical cancer	HPV DNA Detected by PCR E5 variants Detected by restriction enzyme digestion (RFLP) and further characterized by sequencing	ORs for HPV-16 E5 variants and CIN (CIN versus not CIN) Variant 1 1.0 Variant 2 2.6 (0.8–8.2) Variant 5 0.2 (0.1–1.0)	Variant 1 detected at a similar prevalence among those with and without cervical neoplasia

Reference	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Hildesheim *et al.* 2001b Costa Rica	Prevalent case-control study within population-based cohort of 10,077 women who were screened for cervical abnormalities; variants were identified in 176 HPV-16-positive women: 16 cancer 56 HSIL 20 LSIL 84 equivocal or normal lesions	HPV DNA Detected by Hybrid Capture Tube test. PCR also was performed on > 40% of cohort, including women with abnormalities Host genotyping (microsatellite) Performed on 140 women to determine degree of genetic relatedness of study subjects HLA alleles Detected on subset	RRs for HPV-16 LCR variants in HPV-positive population HSIL European prototype 1.0 European-like 0.9 (0.3–2.6) Non-European 2.7 (0.8–9.9) Cancer European prototype 1.0 European-like 0 (0.0–3.5) Non-European 11 (2.5–50) HLA alleles and variants Non-European variants associated with HLA DRB1*1102-DQB1*0301 haplotype; P = 0.0005 LCR – long control region	Previous reported ORs for HPV-16 were 320 (97–1,000) for HSIL and 710 (110–4,500) for cancer Findings not due to cosegregation of variants among subpopulations (i.e., socioeconomic status, genetic relatedness or geography)

Reference	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Cohort studies				
Xi *et al.* 1997 Washington, USA	Two cohorts (123 women): 57 female students enrolled in university (1990–1995) and 66 women presenting to a sexually transmitted disease (STD) clinic (1989–1995) without CIN II – III and who had at least one HPV-16-positive smear; women were followed every 4 months until CIN II or III biopsy, loss of follow-up, or 1995 Cases: 19 HPV-positive women with biopsy confirmed CIN II or III (9 from university and 10 from STD clinic) Controls: 102 HPV-16-positive women who did not develop CIN II or III (46 from the university and 56 from STD clinic)	HPV DNA Amplified from cervical cells by PCR using MY09/11 primers and typed by specific HPV probes Variants Detected by PCR-SSCP and sequencing	Adjusted RR for HPV-16 variants and CIN II-III University 6.5 (1.6–7.2) STD clinic 4.5 (0.9–23.8) Variants nonprototype, prototype assigned RR = 1.0	RR adjusted for age, lifetime number of sexual partners, HPV-16 status at entry, racial or ethnic group and number of visits positive for HPV-16 Elevated risk also observed for variants and CIN II and III when restricted to incident HPV-16 cases

Reference	Study population	Exposure assessment and HPV prevalence cohort or cases/controls	Effects: RR or OR (95% CI)	Comments
Villa *et al.* 2000 São Paulo, Brazil	Ludwig-McGill screening cohort (1993–1997): See Schlecht *et al.* (2001), Table 3.4; average follow-up was 40.9 months Variant analysis performed on 97 HPV-16 isolates (54 subjects) and 25 HPV-18 isolates (12 subjects) 7 types of LCR and 2 additional E6 or L1 variants detected	HPV DNA Detected by PCR using β-globin as control and typed using type-specific probes Virus load Detected by quantitative PCR Molecular variants (LCR) Analyzed by PCR sequencing	Adjusted RR for high-grade SIL and HPV-16 and 18 variants (ref. HPV negative) E branch 6.1 (1.3–27.4) Non-E 22.5 (6.0–83.9) OR for persistent HPV infection and HPV-16 and 18 variants E branch 2.5 (1.3–4.9) Non-E 4.5 (1.6–12.4)	E- European variants (prototype) Non-E- non European variants- AA/Af/As branches and others RRs and ORs adjusted for age and race
CIN progression studies				
Matsumoto *et al.* 2000 Japan	Cohort: 83 women with HPV-16-positive CIN (40 CIN I-III and 43 invasive cervical carcinoma) Follow-up: 17 of the 20 CIN I/II patients every 3–4 months with cytology and colposcopy for up to 33 months (median 31 months) Case/control study: Cases: 43 women with cervical cancer Controls: 40 women with CIN	HPV DNA Detected from exfoliated cells (CIN I/II) or biopsy (CIN III/ICC) by PCR HPV-16 E6 variants Identified by sequencing	Follow-up study: Regression rate of CIN I/II lesions to normal cervix was significantly lower in patients with variants (33%, 4/12) than patients with prototype (80%, 4/5) Risk of cervical cancer and HPV-16 E6 variants (reference group CIN patients) All 4.1 (1.4–12.1) D24E 4.8 (1.4–16.4) L83V 3.0 (0.9–10.5)	Samples from subjects were collected from two hospitals in the same geographic location Small number of patients, especially in follow-up regression analysis D24E (aspartic acid to glutamic acid at codon 24) L83V (leucine to valine at codon 83)

Table A-8. Current cohort and case-control studies on HLA and HPV

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Sanjeevi *et al.* 1996 Västerbotten County, Sweden	Prospective study: Västerbotten cohort (see Chua *et al.* 1996, Table 3.2) Cases: 74 women with invasive cervical carcinoma or SIL Controls: 164 cases without CIN matched for age, sex, and residence	HPV serology HPV-16 capsid antibodies HLA typing DQA1 and DQB1 genes were amplified by PCR and typed using probes or sequence-specific primers	Case/control in HPV-16-positive patients Alleles with positive findings DQA1*0102 3.8 (1.4–10.8) DQB1*0602 5.7 (1.9–17.1)[+] DRB1*15 5.8 (1.9–17.6)[+] Haplotypes DQ6 (DQA1*0102/DQB*0602) 6.0 (2.0–18.1)[+] DR15DQ6 5.8 (1.9–17.6)[+] [+]Also associated with risk in all patients (HPV positive and negative)	Data only given for alleles with significant findings; negative results observed for other alleles
Helland *et al.* 1998 Oslo, Norway	Population case-control study (1991–1992) Cases: 91 women with CIN II-III Controls: 213 women without CIN identified from the population register and matched on age	HPV DNA Detected by PCR using general nested primers and typed using type-specific primers HLA alleles Amplified by PCR and typed using slot-blot analysis with specific probes	ORs for CIN (total cases) HPV 77.3 (30.0–229) HPV-16 28.4 (13.4–62.1) HLA DQA1 or B1 no association ORs HPV-16-positive subjects DQB1*0602 10.1 (1.3–449)[+] DQB1*0604 0.1 (0.0–0.9) (DQA1/DQB1) 0102/0602 10.1 (1.3–449)[+] 0102/0604 0.1 (0.0–0.9) [+]Also increased risk among HPV-positive patients (not restricted to HPV-16)	Data only presented for alleles with significant findings Results for HPV-positive subjects ORs for elevated associations no longer significant when correcting for number of comparisons

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Hildesheim *et al.* 1998 Portland, Oregon United States	Nested case-control study: same population as described by Liaw *et al.*, in Table A-4 but later follow-up Cohort: Kaiser Permanente clinics - 24,000 women Cases: 141 women with high-grade SIL (study also included women with low-grade SIL or HPV infection) Controls: 202 HPV-negative women with normal cytology	HPV DNA Detected by PCR using MY09/11 primers or Hybrid Capture tube test HLA alleles Detected by PCR using specific primers	RR for high-grade SIL in HPV-16-positive patients HLA Class I B7 2.5 (1.2–5.1) HLA Class II antigens DQB1*0302 1.7 (0.8–3.5) DRB1*13 0.6 (0.3–1.3) Haplotypes DRB1*1501/DQB1*0602 0.2 (0.1–0.6) Combined Risks B7 & DQB1*0302 8.0 (2.1–3.1)[+] DRB1*1501/DQB1*0602 and DRB1*13 0.6 (0.1–6.1) HPV-16 cases versus controls (%) DRB1*1501 12.1/23.9; *P* = 0.04 [+]As reported; upper 95% CI is smaller than OR	No synergistic effects for multiple alleles were observed but limited power
Ferrera *et al.* 1999b Tegucigalpa, Honduras	Case-control study Cases: 49 women with CIN, 24 with severe (CIN III or invasive cervical cancer) and 25 with mild (CIN I or II); subset of a large case-control study disease Controls: 75 women with a normal cervix, matched on age and clinic	HPV DNA Detected by PCR using MY11/MY09 primers and identified by type-specific primers HLA alleles Amplified by PCR from DNA isolated from cervical scrapes and typed using type-specific probes	RRs for severe cases/all cases HLA-DQA1*0301 3.5, *P* = 0.008 (reference: controls) HLA-DQA1*0501 0.30, *P* = 0.03 (reference: mild cases) RR for CIN (any)/HPV-16 or 18 positive patients DQB1*0602 0.34, *P* = 0.04	Limitations: Small number of cases to be stratified by disease stage and HPV status RR for CIN in HPV-positive patients based on 6 cases and 9 controls, multiple comparisons

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Maciag and Villa 1999 João Pessao, Brazil	Subjects randomly selected from previous case-control study (1986–1990) from a hospital-initiated screening program Cases: 161 women with cervical cancer newly diagnosed or admitted to hospital for surgery Controls: 257 women with normal Pap smears	HPV DNA Detected by PCR using MY09/MY11 primers and typed by hybridization to specific probes HLA typing Alleles were amplified by PCR from DNA isolated from cervical cells and typed by sequence-specific probes	Adjusted ORs for cervical cases/all patients DRB1*15 2.2 (1.3–3.9) DQB1*05 0.6 (0.4–0.9) DRB1–DQB1 haplotypes DRB1*15/DQB1*0602 2.0 (1.2–3.6) Adjusted OR for cervical cancer/ HPV-16-positive subjects DRB1*03 1.9 (1.0–3.4) DRB1*15 2.3 (1.3–4.2) Adjusted OR for HPV infection DRB1 *0405 6.2 (1.7–23.2) DRB1*15 2.4 (1.0–6.1) DRB1*1503 4.6 (1.3–15.9) DQB1*0602 2.5 (1.1–5.8)	ORs adjusted for age and ethnic groups Only significant associations reported in table Limitations: Multiple comparisons
Krul *et al*. 1999 The Netherlands and Germany	Case-control study Cases: 172 patients with invasive cervical cancer and 116 patients with CIN (1989-1995) Controls: 1,161 healthy, randomly selected unrelated blood donors	HPV DNA Detected by PCR and typed by sequencing HLA alleles A and B were measured by standard microcytotoxicity test and DR and DQ by propidium iodide staining and automated reading methods	RR HLA Class I antigens B63 13.4; $P = 0.02^{+}$ (using cases with HPV types other than 16 and 18) $^{+}$Corrected for number of comparisons	

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Neuman *et al.* 2000 St. Louis, Missouri, USA	Family-based data (nuclear family) to test for association between DQB1 locus and invasive cancer (1986–1997) Population: 96 women with invasive cervical cancer with HLA information on at least one parent Cases: transmission of parental allele in affected offspring Controls: (internal) nontransmitted alleles	HPV DNA Detected from tumors using PCR and typed by restriction enzyme digestion HLA alleles Amplified by PCR using DNA isolated from blood or buccal cells and typed by sequencing or by hybridization using specific probes; genotyping on both patients available 75% of cases	Transmission/disequilibrium test for susceptibility for invasive cancer DQB1 locus/ All patients $P = 0.31$ DQB1 locus/ High-risk HPV $P = 0.006$ DQB1*0303/ High-risk HPV $\chi^2 = 8.0$, $P = 0.005$ HPV and DQB1 alleles No differences in distribution of alleles in HPV-positive and negative patients (test for susceptibility to HPV infection)	Transmission/ disequilibrium: test of linkage disequilibrium, compares number of times a marker allele from a heterozygous parent is transmitted to affected offspring compared to how many times it is not transmitted Multiple comparisons compensated by using likelihood-ratio method with one degree of freedom
Wang *et al.* 2001 Costa Rica	Nested case-control study Cohort: 10,077 women Cases: 36 invasive cancer, 130 high-grade SIL (also included women with low-grade SIL and HPV infected women) Controls: 173 HPV negative women with ≥ 5 sex partners and normal cytology	HPV DNA Detected by PCR using MY09/MY11 primers HLA alleles Detected by PCR using specific primers and labeled probe	OR for high-grade SIL and cancer in HPV-16 positive cases DQB1*0603 0.1 (0.03–0.6) DRB1*1301 0.2 (0.1–0.7) Combined analyses DRB1*1301 and DRB1*0603 0.1 (0.01–0.6) B*07 and DQB1*0302 8.0 (1.6–41)	

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Beskow *et al.* 2001 Uppsala County Sweden	Nested case-control Uppsala County Sweden (see Ylitalo *et al.* 1999, Table A-2) Cases: 440 with carcinoma *in situ* Controls: 476 age-matched	HPV-16 DNA Detected by PCR, Taqman assay HLA PCR with reverse dot blots	% of HLA allele in HPV 16 positive cases vs. controls DQB1*0602 39 vs. 27; $P = 0.028^{+}$ DRB1*1501 40 vs. 28; $P = 0.027^{+}$ $^{+}$corrected for multiple comparisons Association between HLA alleles and HPV 16 infection DRB1*1501 1.7 (1.3–2.3) DRB1*0602 1.5 (1.1–2.0) Long-term infection vs. short-term infection (%) DRB1*1501 carriers 43/30 DRB1*1501 noncarriers 57/70; $P = 0.09$ DRB1*0602 carriers 42/30 DRB1*0602 noncarriers 58/70; $P = 0.12$	
Lin *et al.* 2001 Senegal	Cases: 55 invasive cervical cancer; 35 years or older Controls: 83 HPV-positive and 107 HPV-negative women matched on age; selected from women 35- years old or older at the same clinic	HPV DNA Detected by PCR using MY09/MY11 followed by hybridization to probe HLA alleles Amplified by PCR and detected by hybridization with specific probes giving unique patterns	ORs for invasive cancer DRB1*13 0.5 (0.2–1.1) haplotypes DRB1*1101/DQB1*0301 2.6 (1.0–7.1)	

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Focus: HLA and HPV variants				
Brady *et al.* 1999 England	Cases: 110 patients with cervical carcinoma (stages 1–4) and positive for HPV-16 Controls: cadaver organ donors, HLA from previous study with known HLA (946 HLA-A, 222 HLA-C, 144 HLA-DRB1 and DQB1) and 74 for p53	HPV DNA Detected by PCR from tumor biopsies using general primers and typed using type-specific primers; HPV-16 E6 variants were identified by sequencing (cases only) HLA Typed using serological assay Class I HLA-A, B and in DNA from white cells by PCR (Class II HLA-DRB1, DQB1)	HLA percent cases vs. controls HLA B15 1.3 vs. 13.2, $P = 0.004$ DRB1*07 42 vs. 24, $P = 0.01$ HPV-16 variants and HLA No significant differences in the HLA frequencies between the HPV-16 350T and HPV-16 350G patients populations HPV-16 variants and stage of cancer HPV 350 variant: no significant difference in distribution of variant and stage of disease or in patient survival	HPV-16 350T considered to be the prototype

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Bontkes *et al.* 1998 The Netherlands	Non-intervention follow-up study of 352 women with CIN – same cohort as Remmink *et al.* 1995 (see Table 3.3) 88 HPV-16-positive women PROG: 8 women with CIN progression NPROG: 80 women with stable or regressing lesions 30 women who remained HPV negative (NEG) Population used for susceptibility analyses 30 HPV-negative and 88 HPV-positive women	HPV DNA Detected by PCR using GP5+/GP6+ primers and typed by specific primers (6, 11, 16, 18, 31 and 33); variants analyzed by sequencing from 40 patients HLA alleles Typed by PCR and sequence-specific probes	ORs for disease progression in HPV-positive women HLA–B44 PROG vs. NEG 12.0 (5.7–25.4), $P = 0.16$ PROG vs. NPROG 9.0 (4.6–17.5), $P = 0.18$ ORs for susceptibility to HPV infection (HPV-16 positive versus negative patients) HLA-DRB1*07 5.9 (3.0–11.3); $P = 0.29$ HLA-DRB1*07 and HPV 350 T/G variant ORs 4.3 (1.5–27.3); $P = 0.08$ HPV-16 E6 and E7 variants No significant association of the 350 T/G mutation with persistent HPV-16 infection, progression or high grade lesions	*P* values corrected for number of alleles compared due to small sample size *P* value corrected for number of comparisons Limitations: Small number of cases

Reference	Study population	HPV detection and identification of genetic factors	Effects: RR or OR (95% CI) or other measures	Comments
Cuzick *et al.* 2000 England and Scotland	Cases: 116 cervical scrapes from women with cervical cancer prior to treatment Controls: 155 cervical scrapes from normal women who were negative for high-risk HPV	HPV DNA Detected and typed by PCR using consensus and type-specific primers (16, 18, 31 and 33); HPV-16 E variants were identified by sequencing HLA alleles Amplified by PCR and typed by sequencing	ORs for cervical cancer risk (ORs), significant associations All cancers DQB1*0301 1.7 (1.1–2.7) DQB1*0501 0.5 (0.3–1.0) haplotype (DRB1*/DQB1*) 0401/0301 2.4 (1.1–5.2) High-risk HPV-positive cancers DQB1*0301 2.5 (1.3–4.9) [+] DQB1*1501 1.7 (1.0–3.0) [++] DRB1*0401 3.4 (1.6–7.3) [+] Haplotype (DRB1*/DQB1*) 0401/0301 4.8 (1.8–12.2) [+] 1501/0602 1.8 (1.0–3.3) [++] HPV-16 T350G variant No increased risk HPV-16 variants and HLA HPV-16 T350G – T variant more frequent (nonsignificant) in women with DRB1*1501, DQB1*0602 and haplotype 1501/0602	[+] High-risk HPV other than HPV-16 [++] HPV-16

Table A-9. Additional studies on cofactors

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Serology and DNA				
Olsen *et al.* 1996, Olsen *et al.* 1998 Norway 1998 (focus on smoking and HPV-16)	Population case-control study (1991–1992) Cases: 90 women with CIN II-III detected by screening Controls: 216 women randomly selected from population and matched for age	HPV serology HPV-16 capsid antibodies HPV-16 seroprevalence 34/16 HPV DNA Detected from smears by PCR using general nested primers detecting 6, 11, 16, 18, 31, and 33 HPV-16 DNA prevalence 67/7	ORs for HPV and CIN Age-adjusted / Adjusted for age and other variables DNA 16: 157 (54–457) / 221 (60–811) Others: 42 (15–121) / 52 (16–174) Serology: 2.8 (1.6–5.1) / 2.0 (0.7–5.6) Cofactors Smoking: 4.1 (2.0–8.2) / 1.1 (0.4–3.6) 10 + Partners: 8.6 (3.5–21.3) / 8.8 (2.0–39) < 17 first intercourse: 2.9 (1.4–5.8) / 1.6 (0.5–5.4) Sensitivity of HPV serology as measured by HPV-16 DNA Controls 14%, cases 42% ORs for smoking and disease: (1998) Seronegative/no: 1.0 (ref) Seronegative/yes: 4.4 (1.8–10.9) Seropositive/no: 2.9 (0.7–11) Seropositive/yes: 15.3 (5.3–44.1) DNA-/no: 1.0 (ref) DNA-/yes: 2.2 (0.8–5.6) DNA+/no: 15.7 (3.2–76.5) DNA+/yes: 66 (22.3–194)	ORs adjusted for HPV DNA, HPV serology, age at first intercourse, number of sexual partners, smoking habits, and educational level 1998 analysis ORs for smoking adjusted for age

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Kjellberg *et al.* 1999 Västerbotten County, Sweden	Population case-control study (1993–1995) Cases: 250 women with pathological cervical smear (various grades); 174 identified from cervical screening programs and 76 others Controls: 320 women randomly selected from screening program; matched for age and years of residence	HPV serology Capsid antibodies HPV seropositive prevalence CIN III/CIN II/Con 16 50/32/26 18 31/11/17 33 30/24/14 HPV DNA Same as in Kjellberg *et al.* 2000; only used in present study for validation	ORs for cervical disease and HPV serology CIN II / CIN III 16 1.3 (0.6–2.8) / 2.8 (1.7–4.6) 18 0.6 (0.1–1.7) / 2.2 (1.2–3.8) 33 1.9 (0.8–4.5) / 2.6 (1.4–4.7) HPV serology sensitivity (using HPV DNA as a standard) DNA: 16 (65%), 18 (69%), 33 (75%) Cofactors: ORs for sexual history (5 + partners and CIN (II and III) Total population 4.4 (1.6–15.1) HPV seropositive (16, 18, or 33) 1.2 (0.3–8.0)	HPV seropositivity strongly related to lifetime number of sexual partners
Kjellberg *et al.* 2000 Västerbotten County, Sweden	Population case-control study (1993–1995) same as Kjellberg *et al.* (1999) Cases: 137 women with high grade CIN (II and III) Controls: 253 women selected randomly from population registry with normal cytology, matched for age	HPV serology Same as Kjellberg *et al.* (1999) HPV DNA Detected from cervical samples by PCR with consensus primers and typed using specific primers for 11, 16, 18, and 33	Combined effects of HPV and smoking HPV/smoking Seronegative/no 1.0 (ref) Seronegative/yes 5.6 (2.6–12.0) Seropositive/no 5.2 (2.5–11.0) Seropositive/yes 10.5 (5.0–22.4) DNA-/no 1.0 (ref) DNA-/yes 4.3 (1.5–12.7) DNA+/no 110 (37–326) DNA+/yes 189 (65–552)	Risk factors considered: sexual history, oral contraceptives and pregnancy; a single pregnancy was still significant after adjusting for HPV infection

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Serology				
Madeleine *et al.* 2001 Washington state, USA	Population case-control study in Washington state (ACIS of the cervix) (1990–1996) Cases: 150 women diagnosed with ACIS identified by cancer registry (SEER) Controls: 651 women identified by random digit dialing frequency matched for age	HPV serology HPV-16 and 18 capsid antibodies HPV seroprevalence 16 23/30 18 51/25 HPV DNA Detected from biopsy (cases only) by PCR using L1 consensus primers and E6 type-specific primers; confirmed by Southern blot analysis and typed by restriction enzyme digestion HPV DNA prevalence Cases 87	Adjusted ORs for ACIS HPV-16 antibody 0.7 (0.5–1.1) HPV-18 antibody 3.3. (2.2–4.9) HSV-2 antibody 1.2 (0.8–1.9) History of genital warts 1.5 (0.9–2.5) 5 + partners 1.8 (1.1–3.1) < 18 first intercourse 1.7 (1.1–2.8) Smoking 0.8 (0.5–1.3) Oral contraceptive (born later than 1945) ever used 2.7 (1.2–5.8) Dose/response (test for trend): duration of use, ($P < 0.001$), age at first use ($P = 0.02$), interval since last use ($P < 0.001$)	ORs in total population adjusted for age, except for history of warts and smoking, which were adjusted for age and number of partners ORs for oral contraceptive use adjusted for age, lifetime number of partners, interval since last Pap smear

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
DNA				
Kjær 1998 Copenhagen, Denmark	Population case-control study; subjects were identified at enrollment; an ongoing prospective study (11,088 women 1991–1993) Cases: abnormal Pap smear at enrollment (131 ASCUS, 120 LSIL and 79 HSIL) Controls: 1,000 women randomly chosen with normal cytology	HPV DNA Detected by PCR using GP5+/6+ primers and typed by Southern blot analysis using probes specific for 6, 11, 16, 18, 31 and 33 HPV DNA Prevalence Controls 16 ASCUS 65 LSIL 72 HSIL 90	RR for HPV detection and CIN ASCUS 9.3 (6.2–14.1) LSIL 13.9 (8.9–21.6) HSIL 50.3 (23.5–107.7) Cofactors: main risk factors in HPV positive women HSIL: years of sex life without barrier contraceptive, smoking, early age of first genital warts ASCUS and LSIL: smoking, parity, and number of sex partners ASCUS – atypical squamous cells of undetermined significance LSIL – low-grade SIL HSIL – high-grade SIL	Risk factors considered: number of sex partners, age at and years since first intercourse (separately), age at first episode of genital warts, years of sex without barrier contraceptive, oral contraceptive use, history of *Chlamydia*, year of birth of first male partner, parity, and smoking

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Hildesheim *et al.* 2001a Costa Rica	Same cohort as Herrreo *et al.* 2000 (above) but study restricted to HPV-positive women Cases: 146 HPV-positive women with HSIL (116) or cervical cancer (CA) (30) Controls: 843 HPV women with (140) and without (703) LSIL at entry into cohort	HPV DNA Detected using the Hybrid Capture Tube test for 16 types and by PCR (on a subset [2,974] of the original cohort) for 44 types	Adjusted RR and AF (attributable fraction) for HSIL/CA in women positive for high risk HPV RR / AF (%) > 3 pregnancies 2.2 42 Cigarette smoking 2.1 10 > 3 pregnancies and/or smoking 2.2 44 Risk increased with increasing number of pregnancies, (P_{trend} = 0.12) and live births, (P_{trend} = 0.04) and with increasing number of cigarettes/day, (P_{trend} = 0.003) Other significant associations (adjusted RR) Barrier contraceptives 0.39 (0.16–0.97) Risk factors considered but not found to be significant include sexual behavior, oral contraceptives Combined effects: pregnancies and OC use in HPV (all)-positive women (age-adjusted RR) OC / < 3 pregnancies / > 3 pregnancies Never 1.0 4.7 (1.8–12) < 5 years 1.8 (0.65–4.9) 3.7 (1.5–9.3) < 5 years 3.1 (1.1-9.1) 4.0 (1.5–10)	RR adjusted for age and pregnancy (cigarette smoking and barrier contraceptive) or cigarette smoking (pregnancy and barrier contraceptive) Limitations: HPV-positive controls may have been biased towards women with newly acquired HPV, which may have influenced sexual behavior, or women with persistent infection, which may have attenuated finding

Reference	Study population	Exposure and HPV prevalence (cases/controls)	Effects: RR or OR (95% CI)	Comments
Castle *et al.* 2002 Portland, OR	Prospective cohort study Kaiser Permanente cohort (see Liaw *et al.* 1999 – Table A-2) – 23,702 women (1989-1990) 10-year follow-up of 1,812 women who were HPV positive at enrollment; median number of Pap smears = 4 Cases: 68 women (58 with CIN III and 10 with cancer) with questionnaire data Controls: 926 (898 who never became cases and 28 who were matched to early cases and later became cases)	HPV DNA Detected from cervicovaginal lavages using the Hybrid Capture 2 probe B microplate assay (13 high-risk types) Cofactors Smoking habits, oral contraceptives, and a history of parity were assessed by a short enrollment questionnaire	RR for CIN III and cancer Smoking (≥ 1 pack/day) 2.9 (1.5 –5.6) Parity (≥ 3) 0.7 (0.3–1.6) Oral contraceptive use 0.8 (0.5–1.5) Multivariate analysis – OR for CIN III and cancer Smoking (former) 3.3 (1.6–6.7) Smoking (< 1 pack/day) 2.9 (1.4–6.1) Smoking (≥ 1 pack/day) 4.3 (2.0–9.3) Parity (≥ 3) 0.7 (0.2–1.9) Oral contraceptive use 0.6 (0.3–1.1)	Multivariate models were adjusted for age, underlying prevalent disease and screening patterns ORs were calculated using conditional logistic regression Cases were matched to controls by cytology of base-line smear, age at enrollment, number of Pap smears during follow-up, and age at both the prediagnosis visit and at diagnosis Population was a low-parity population compared to international studies

Table A-10. Current studies in HIV populations

Reference	Cancer type	Study design and population	Measurement of exposure and HPV prevalence (cohort or cases/controls)	Effects: RR or OR (95% CI) and other measures	Comments
Frisch *et al.* 2000 USA	Various HPV-associated cancers	Prospective study AIDS-Cancer Match Registry (1995–1998) 309,365 US patients with HIV infection/AIDS were followed from 5 years before onset of AIDS to 5 years after onset, 1,586 HPV-related cancers (mainly anogenital); 1,154 women and 432 men	HPV Not measured Number of observed invasive cancers Cervical 44 Vulvar/vaginal 12 Anal/women 7 Anal/men 214 Penile 14 Tonsillar/men 29	RRs for period 60 months before to 27 months after AIDS onset except invasive cervical carcinoma (4–27 months after AIDS onset) Cervical Invasive 5.4 (3.9–7.2) *In situ* 4.6 (4.3–5.0) Vulva/vagina Invasive 5.8 (3.0–10.2) *In situ* 3.9 (2.0–7.0) Anus (women) Invasive 6.8 (2.7–14.0) *In situ* 7.8 (0.2–43.6) Anus (men) Invasive 37.9 (33.0–43.4) *In situ* 60.1 (49.2–72.7) Penis Invasive 3.7 (2.0–6.2) *In situ* 6.9 (4.2–10.6) Tonsillar Men 2.6 (1.8–3.8) RR and AIDS onset RRs increased over time (pre-to post-AIDS) for all *in situ* cancers (cervical, vulvar/vaginal and penile) and penile invasive cancer but not for other invasive cancers (vulvar/vaginal or anal).	RR calculated from observed vs. expected number of cancers for different time periods in relation to AIDS onset; invasive cervical cancer by definition (on AIDS onset) could not occur in pre-AIDS periods, so trend test could not be done Large-study population

Reference	Cancer type	Study design and population	Measurement of exposure and HPV prevalence (cohort or cases/controls)	Effects: RR or OR (95% CI) and other measures	Comments
Cappiello *et al.* 1997 Italy	Cervical neoplasia	Multicenter study, (1994–1995), 232 subjects cross-sectional analysis 134 HIV-positive women, 58 with SIL 98 HIV-negative women, 9 had SIL	HPV DNA Detected from cell smears by PCR using MY09/MY11 primers and typed by restriction enzyme digestion HPV prevalence SIL+/HIV+ 76 SIL+/HIV- 67 SIL-/HIV+ 21 SIL-/HIV- 26	ORs for HPV-associated SIL in HIV positive and negative women Any 7.7 (2.4–25.7) HR 3.0 (1.4–6.7) LR 1.4 (0.3–5.9) HR (high risk): 16, 18, 31, 33, 35, 54, 58, 59, 66, LR (low risk): 11, 44, 53	ORs calculated using only HPV-positive women The frequency of HPV-positive SIL cases was compared to HPV-positive controls (or total HPV women for high and low risk) in HIV-infected and noninfected individuals Estimates are the risk for HIV-infected compared to noninfected women Limitations: Risk estimates based on small numbers

Reference	Cancer type	Study design and population	Measurement of exposure and HPV prevalence (cohort or cases/controls)	Effects: RR or OR (95% CI) and other measures	Comments
La Ruche *et al.* 1998, La Ruche *et al.* 1999 Abidjan, Côte d'Ivoire, Africa	Cervical neoplasia persistence	Multicenter study of three clinics (1995–1996) 1998: three case-control studies LSIL: 151 cases and 151 controls HSIL: 60 cases and 240 controls Invasive cancer: 13 cases and 65 controls 1999 prospective study: 94 of the 151 LSIL cases who returned to clinic for follow-up smear, HPV detection at enrollment and follow-up smear available for 45 (for persistent HPV infection) 39 cases of persistent SIL developed	HPV DNA Detected by PCR using MY09/MY11 primers and typed by restriction enzyme digestion 16 related 16, 31, 33, 35, 52, 58, 67 18 related 18, 39, 45, 59, 68, and 70 HIV Detected by serology	ORs for HSIL and viral infections HPV-16/33/18 (HR)/HIV-1 HR-/HIV+ 2.3 (0.4–10.1) HR+/HIV- 101 (16.4–1,010) HR+/HIV+ 132 (22–1,277) Prevalence of HPV genotypes Significant associations: 16, 31, 33, 52 (borderline), 58, 67, 18, 70, 56 No associations 35, 39, 45, 59, 68, 6, 11, 62 Adjusted OR (multivariate) for invasive cancer HPV 13.3 (3.2–55.5) HIV-1 no association Persistence of SIL study (1999) RRs for persistence of SIL and viral infections HPV enrollment/HIV-1 HPV-/HIV+ 5.3 (1.1–24.5) HPV+/HIV- 2.1 (0.5–9.4) HPV+/HIV+ 8.4 (2.2–31.8) Persistent HPV/HIV HPV-/HIV+ 4.3 (0.4–51.3) HPV+/HIV- 3.3 (0.4–25.6) HPV+/HIV+ 10.0 (1.5–67.3) HPV genotypes: only HPV-56 significantly associated with persistent SIL	LSIL: Similar patterns but lower risk estimates HPV subtypes: HPV-16 related – similar pattern to HPV-16, but OR have lower magnitudes HPV-18 related – similar pattern to 16/33/18, but ORs have lower magnitudes 1999 study: multivariate analysis – HPV not significantly (2.6 [0.7–9.8]) associated with persistent infection after controlling for HIV HPV (either at enrollment or persistent HPV) was no longer associated with persistent SIL after adjusting for HIV status

Reference	Cancer type	Study design and population	Measurement of exposure and HPV prevalence (cohort or cases/controls)	Effects: RR or OR (95% CI) and other measures	Comments
Ellerbrock *et al.* 2000 New York and New Jersey, USA	Cervical neoplasia	Prospective cohort (1991–1996) of women from AIDS, STD, and methadone clinics; 328 HIV infected and 325 uninfected women, followed for 30 months Cases: 83 incident SIL (67 HIV and 16 uninfected)	HPV DNA Detected from cervicovaginal lavages by PCR using consensus primers and typed by restriction digestions HPV prevalence HIV+/HIV- 54/32	Adjusted RR for SIL HPV Transient/ (any type) 5.5 (1.4–21.9) Persistent/ not 16/18 7.6 (1.9–30.3) 16/18 11.6 (2.7–50.7) HIV 3.2 (1.7–6.1) HPV and HIV at enrollment HPV significantly higher in HIV-infected than uninfected women	RR adjusted for HIV, smoking, HPV, age, age at first intercourse, lifetime sex partners, intravenous drug use, history of STD, and socioecomomic status

Reference	Cancer type	Study design and population	Measurement of exposure and HPV prevalence (cohort or cases/controls)	Effects: RR or OR (95% CI) and other measures	Comments
Ahdieh *et al.* 2000 Maryland, USA	Cervical lesions	Prospective ALIVE (AIDS Link to Intravenous Drug Experience) cohort Injection drug users (1988–1989) with no history of AIDS at recruitment, subset of 84 HIV-negative and 184 HIV-positive women who agreed to baseline exam (1992–1997) and further follow-up, (median number of visits = 6) 107 women had colposcopies, 96 were normal, 11 had CIN	HPV DNA Detected from cervicovaginal lavage by PCR using MY09/MY11/HB01 primers and typed with specific probes High-risk HPV: 16, 18, 31, 45 HIV Detected by serology and confirmed by Western blot	Repeated HPV positivity (HPV positive at every visit) % HPV full positivity HIV negative 26 HIV positive CD4+ ≥ 200 cells/μL 59 CD4+ < 200 cells/μL 84 High-risk HPV clearance incidence (Relative Hazard) (reference HIV negative) CD4+ ≥ 200 cells/μL 0.37 CD4+ < 200 cells/μL 0.26 ORs for HIV and CIN in 107 women who had colposcopies HIV 1.6, $P = 0.014$ Stratification and adjustment for repeated positivity, $P = 0.648$ HPV repeated positivity was related to CIN in a dose/response relation (test for trend, $P < 0.001$)	HPV repeated positivity analyzed using logistic regression models for correlated observations HPV clearance measured by survival analysis methods