NTP
National Toxicology Program

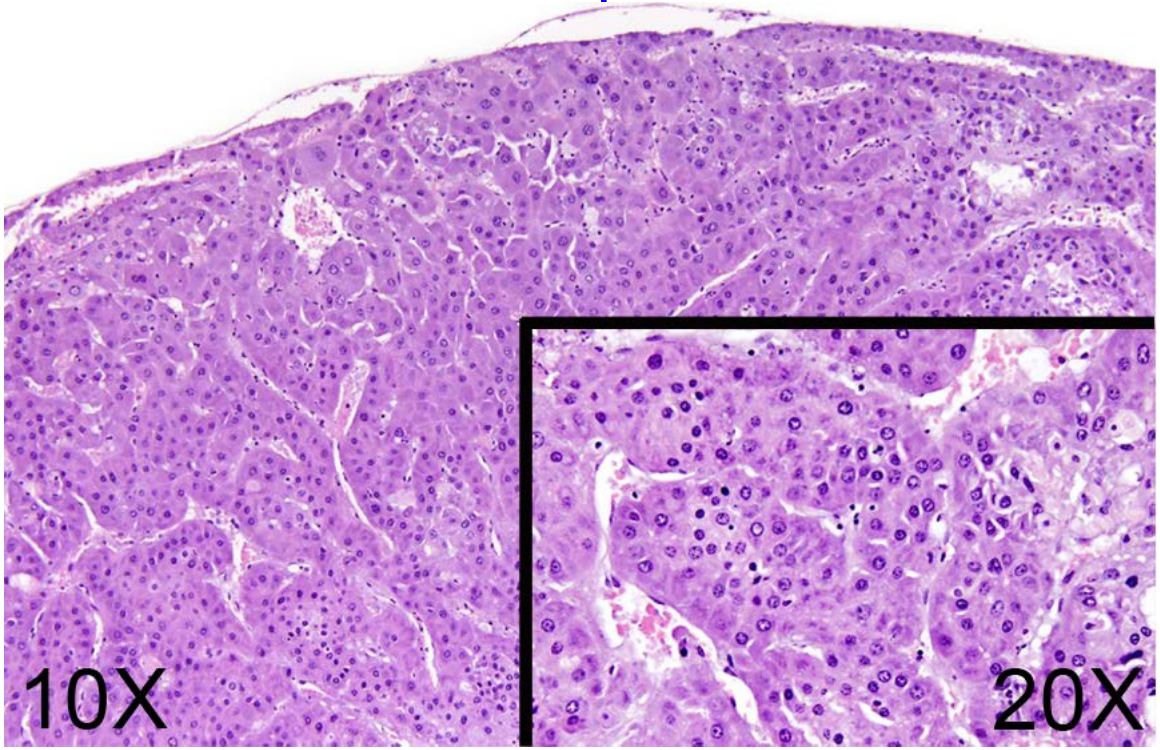
Molecular Studies on *Ginkgo biloba* Extract (GBE) TR 578

Mark J. Hoenerhoff, DVM, PhD, DACVP
National Institute of Environmental Health Sciences

NTP Technical Reports Peer Review Meeting
February 8-9, 2012

DEPARTMENT OF HEALTH & HUMAN SERVICES • USA

NTP 2yr oral gavage study B6C3F1 mice
Dose-dependent increase in hepatocellular carcinoma (HCC)



2yr bioassay	Males				Females			
GBE dose (mg/kg)	0	200	600	2000	0	200	600	2000
Hepatocellular carcinoma	22 (44%)	31* (62%)	41* (82%)	47* (94%)	9 (18%)	10 (20%)	15 (30%)	44* (88%)

*$p < 0.05$

Overview

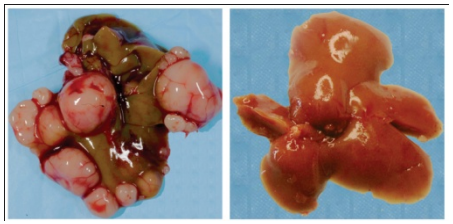
- Mouse & human HCC share important molecular alterations:
  - *H-ras* mutation:
    - Common in spontaneous HCC in mice, induced by chemical treatment (*Watson et al, 1995*)
    - Associated with increased tumor invasiveness in human HCC (*Zhou, 2002*)
  - *β-catenin* mutation:
    - Exon 2 (mice): early event in chemically induced hepatocarinogenesis *(Devereux et al, 1999*)
    - Exon 3 (human): Common alteration in human hepatocarcinogenesis (*de La Coste et al, 1998*)
  - Wnt/β-catenin pathway upregulation:
    - Altered in mouse and human tumors
    - Wnt/β-catenin target genes play a role in:
      - Proliferation (CCND1), Differentiation (MYC), Survival (BIRC5)

Objectives

Compare spontaneous HCC to HCC in GBE exposed mice for:

1. Relevant mutations in mouse and human HCC:
   - Mutation analysis: *β-catenin, H-ras*, *Tp53*
2. Alterations in common HCC pathway expression
   - Wnt/β-catenin pathway: Western blotting, immunohistochemistry
3. Differences in global gene expression profiling
   - Define potential mechanisms of tumorigenesis in GBE exposed animals

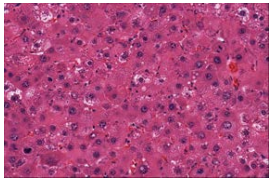
Methods



Fixed tissue

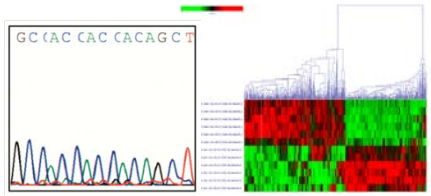
Frozen tissue

Histopathology



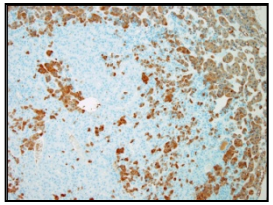
NTP Pathology

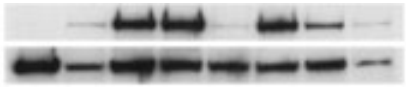
Nucleic Acids



Mutation analysis
Microarray
QRT-PCR

Protein



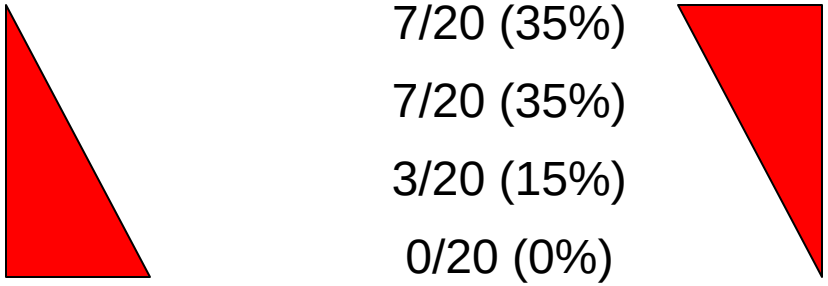


IHC
Western blot

Results: Mutation analysis

Incidence of *β-catenin* and *H-ras* mutation in spontaneous and GBE treated HCC

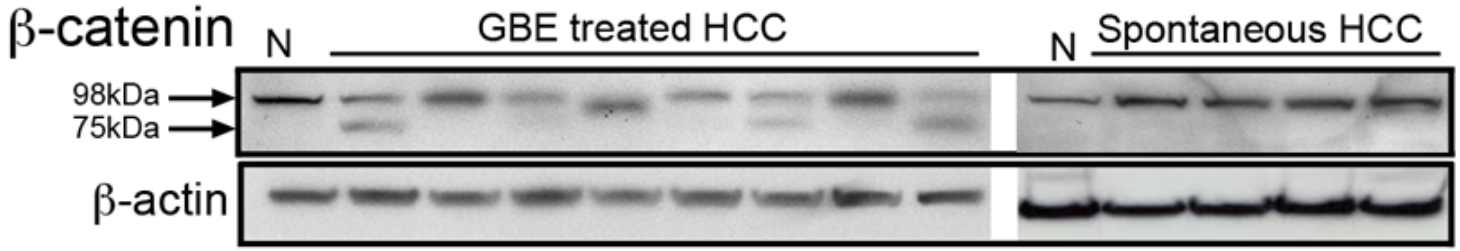
	β-catenin	*H-ras*
Historical Spont	1/59 (2%)[£]	260/473 (55%)[¥]
0mg/kg	0/20 (0%)	7/20 (35%)
200mg/kg	4/20 (20%)	7/20 (35%)
600mg/kg	2/20 (10%)	3/20 (15%)
2000mg/kg	13/20 (65%)	0/20 (0%)



[£]Historical database for β-catenin mutation in spontaneous HCC (Hayashi et al., 2003)
[¥]Historical database for H-ras mutation in spontaneous HCC (Sills et al., 1999 and Maronpot et al., 2005)
Trend analysis for *β-catenin*, $p < 0.00001$; *H-ras*, $p < 0.0075$

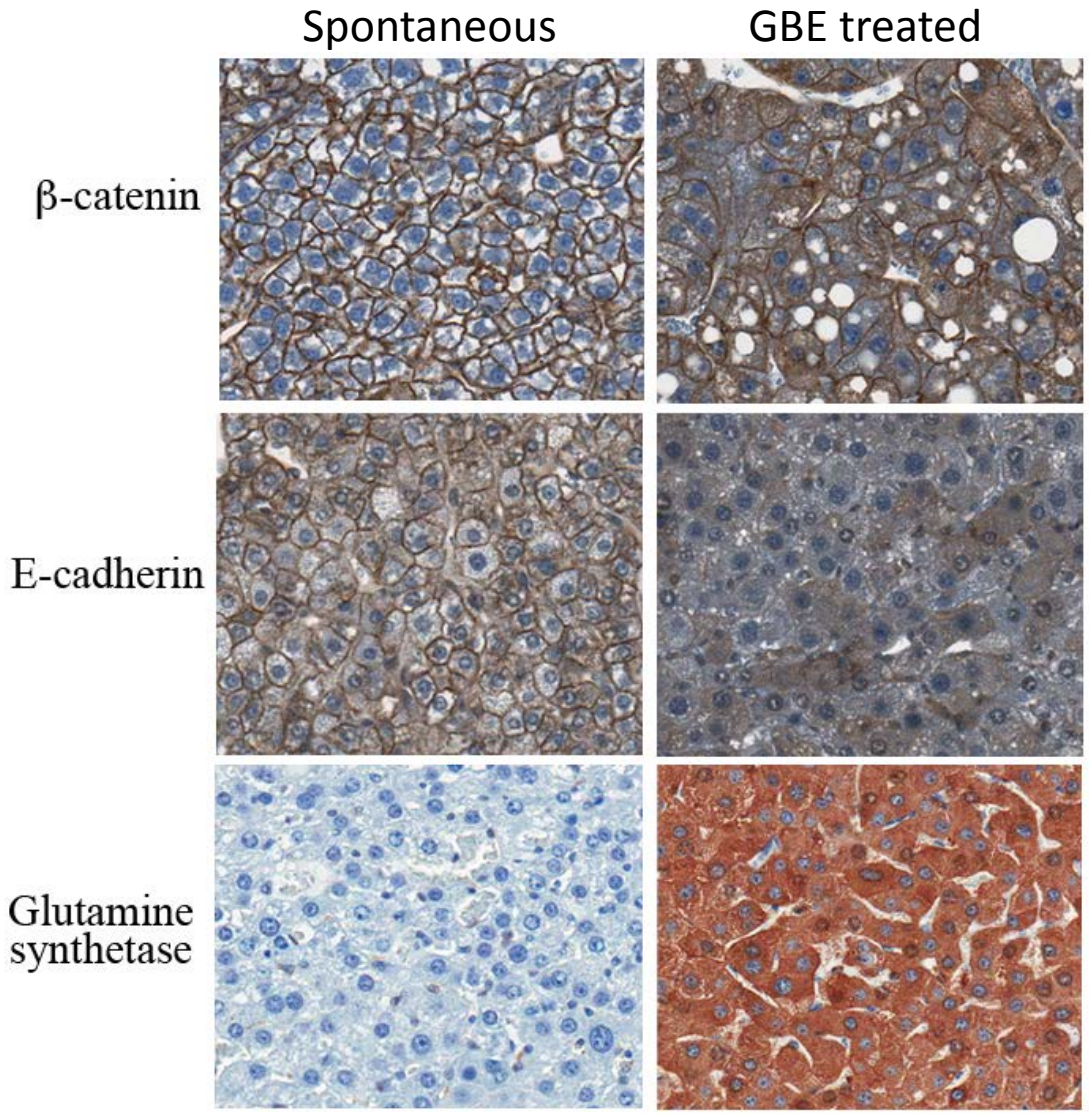
- Increasing incidence of *β-catenin* mutation with dose ($p < 0.0001$)
  - Multiple mutations per animal in high dose, increased deletion mutations
- Decreasing incidence of *H-ras* mutation with dose ($p < 0.0075$)
- No *Tp53* mutations observed

Results: Protein analysis



- β-catenin – 98kDa protein
- GBE treated HCC – second 75kDa band in ~50% of samples
  - Not observed in spontaneous HCC

Results: Protein analysis

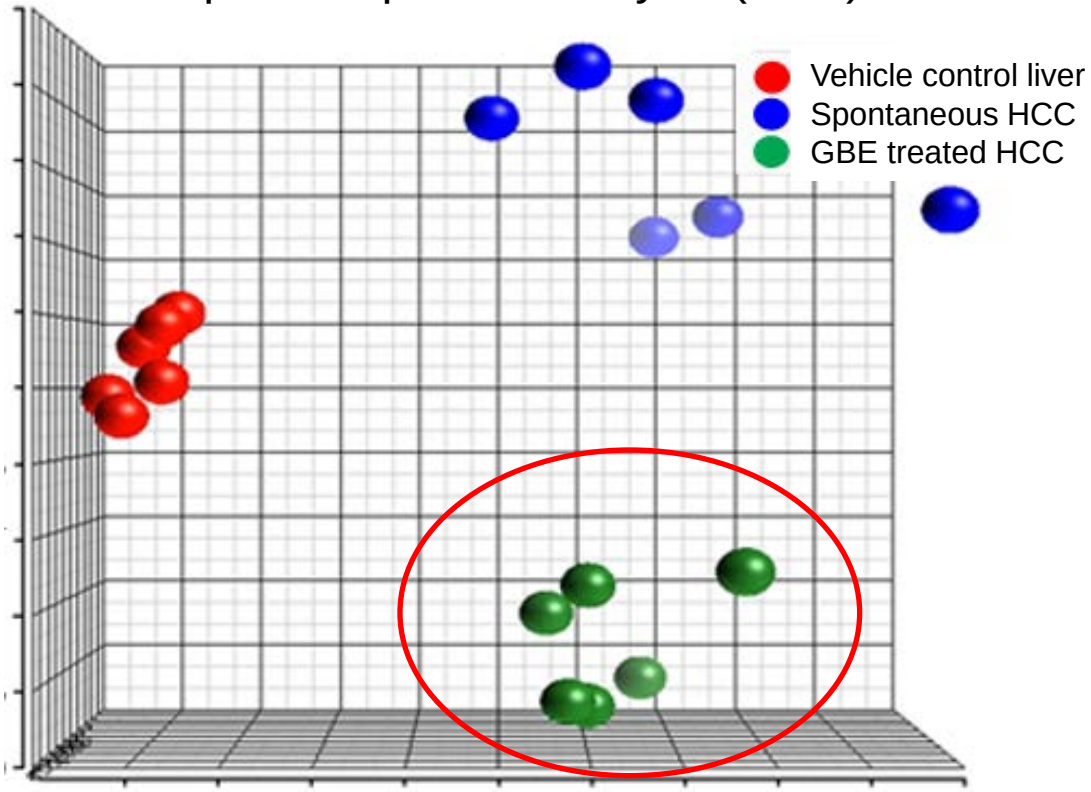


Results: Microarray analysis

- Experimental Groups:
  - Vehicle control normal liver
  - Spontaneous HCC
  - GBE treated HCC
- Affymetrix gene arrays – global gene expression analysis
  - 40,000 transcripts ~ 20,000 genes
- Comparison analysis – Ingenuity Pathways Analysis (IPA)
  - Genes and pathways overrepresented in GBE treated HCC

Results: Microarray analysis

Principal Component Analysis (PCA)

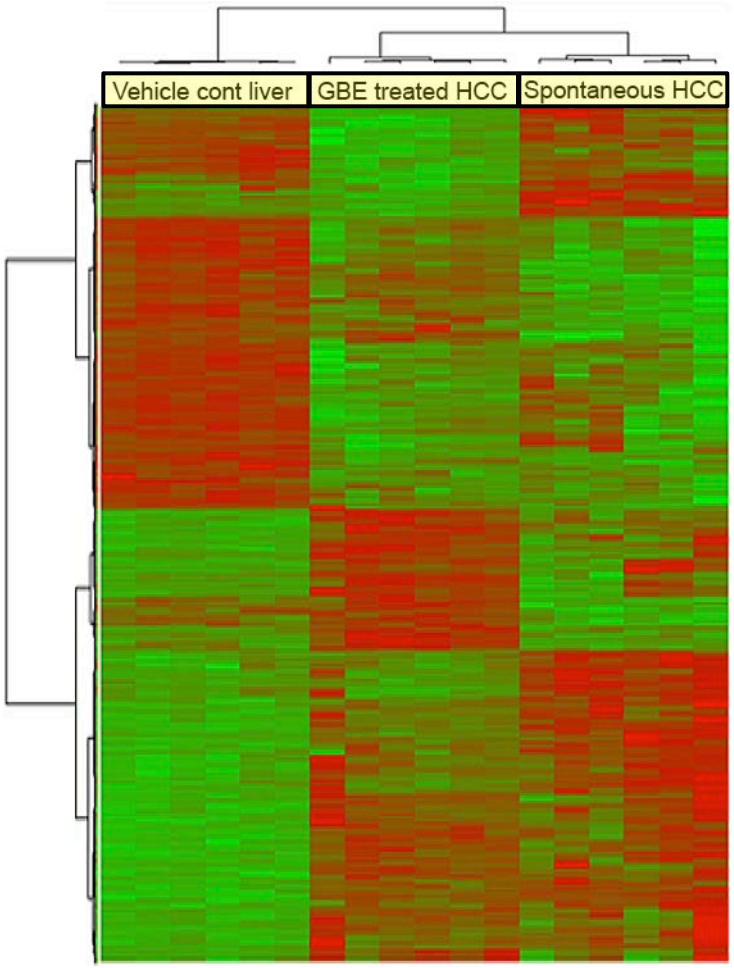


Distinct clustering of samples within groups

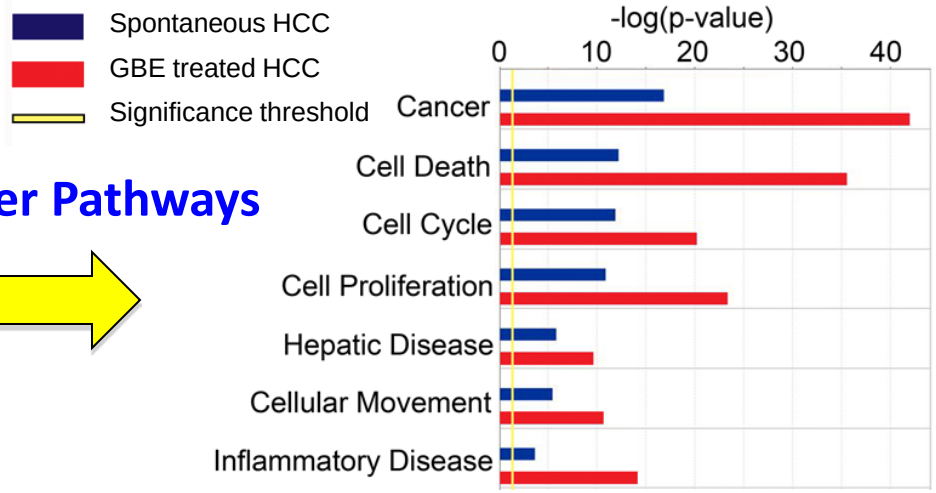
GBE treated HCC cluster separately from spontaneous HCC

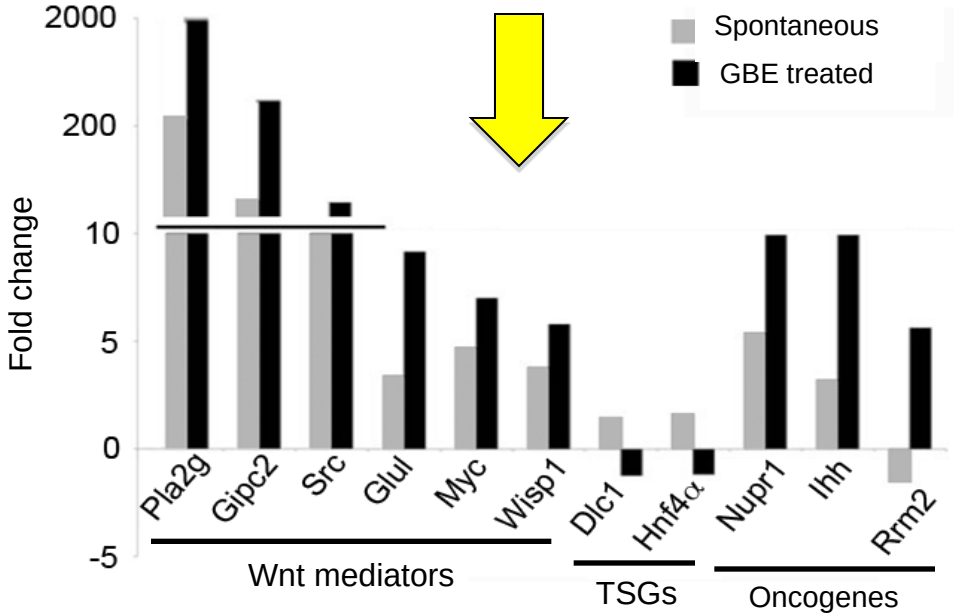
Changes in global gene expression related specifically to GBE treatment

Results: Microarray analysis



Cancer Pathways





Summary

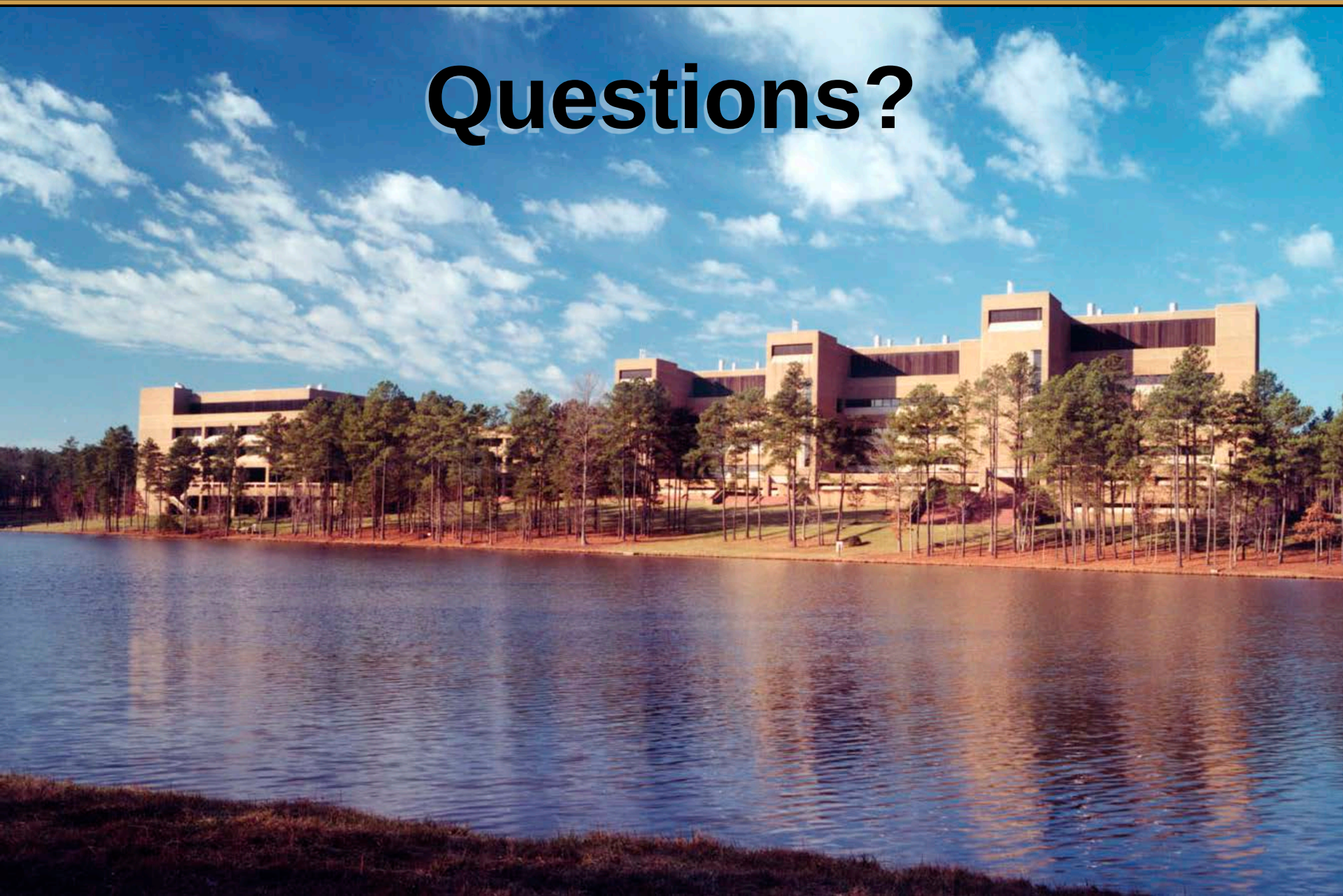
- GBE treated HCC are molecularly different than spontaneous HCC
- Marked increase in *β-catenin* mutation
- Low incidence of *H-ras* mutation
- Alteration in β-catenin protein expression
  - Abnormal localization (β-catenin, E-cadherin) and overexpression (GLUL)
  - Possible transcriptionally active modification associated with human cancer (*Rios-Doria et al 2004, Benetti et al 2005*)
- Marked differences in global gene expression profiling
  - Overrepresentation of cancer pathways
  - Alterations in TSGs and oncogenes found in human HCC

Conclusions

- GBE hepatocarcinogenesis in B6C3F1 mice is a complex process
- Involvement of multiple different pathways and genetic alterations which reflects the complex nature of the compound
- GBE treated tumors exhibit genetic alterations and pathway dysregulation that are known to influence HCC development in both mice and humans

Acknowledgements

- Molecular and Investigative Pathology
  - Robert Sills
  - Arun Pandiri
  - Lily Hong
  - Kiki Ton
  - Stephanie Lahousse
- Microarray Core
  - Kevin Gerrish
  - Laura Wharey
- Bioinformatics/Statistics
  - Shyamal Peddada
  - Keith Shockley
  - Pierre Bushel
- NTP Study Scientists
  - Cynthia Rider
  - Po Chan
- NTP Pathologist
  - Abraham Nyska
- Histology/IHC laboratories
- Protein characterization core
- TGMX Faculty
- NTP toxicologists
- CMPB pathologists



Questions?