Sex-biased gene expression and pathway activation in hepatitis-associated HCC

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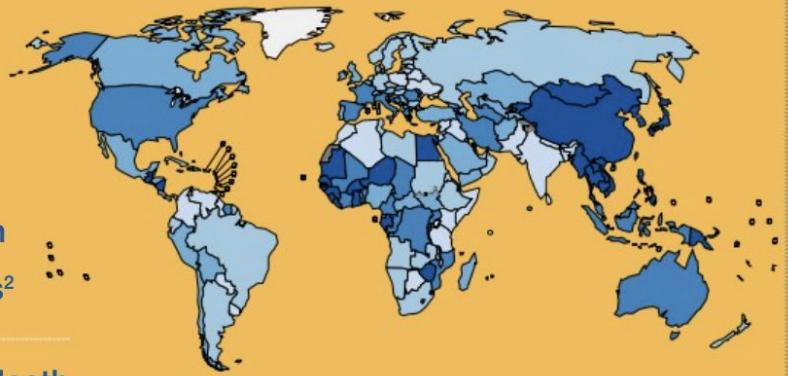
Viral-mediated Hepatocellular Carcinoma (HCC) poses a considerable health problem worldwide

Liver cancer is a global burden:[1]

The 6th most common cancer[2]

The 3rd most common cause of cancer deaths[2]

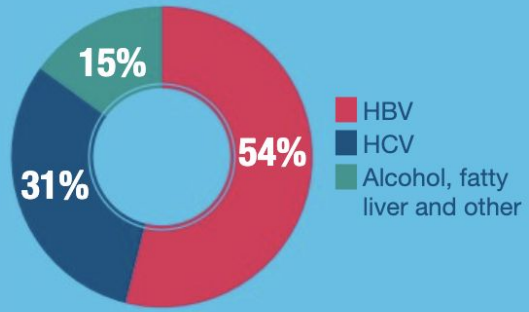
The leading cause of death in people with cirrhosis[3]



ASR (World) per 100,000*

≥ 8.9 | 6.1–8.9 | 4.8–6.1 | 3.7–4.8 | < 3.7 | Not applicable | No data

Most liver cancer is caused by viral hepatitis:[3]



There are substantial regional variations. Fatty liver disease will be increasingly important as it becomes more common[4]

*Taken from Liver Cancer Explained | EASL Campus

Males have higher incidence and worse prognosis with HCC

- The incidence of HCC in males is two to four times that of females
- Males are two times more likely to die from chronic liver disease than females
- Sex differences in HCC remain even after adjusting for risk factors such as alcohol intake and smoking

Taken from Bristol Myers Squibb 2021

Known sex-differences in gene expression in HCC

- Observed sex-specific differences in gene expression across tumor:tumor-adjacent tissues
  - Account for inherent sex differences
  - Did not account for viral etiology
- We don’t know the sex-differences and pathway differences in viral-mediated HCC

Goal: Determine sex differences in differentially expressed genes and pathways in viral mediated liver cancer

1. Identify differentially expressed genes
2. Identify enriched pathways in differentially expressed genes

Whole transcriptome sample dataset

- Whole transcriptome (RNA-seq) data from 260 donors obtained from International Cancer Genome Consortium

	Male Tumor	Male Tumor-adjacent	Female Tumor	Female Tumor-Adjacent
No hepatitis	25	25	3	3
HBV	33	40	8	9
HCV	59	72	34	36
HBV & HCV	4	4	0	0
Total	**121**	**140**	**8**	**9**

*LIRI-JP dataset obtained with controlled access from Dr. Kenneth Buetow

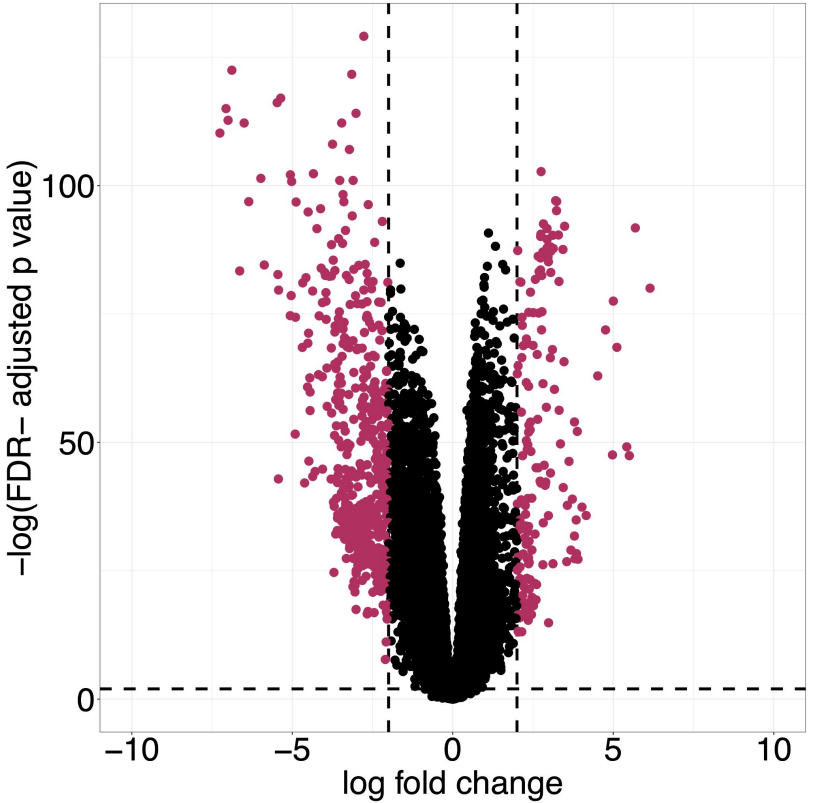
Genes are differentially expressed in HCC

Differentially expressed genes→ logFC ≥ 2 and p.value < 0.05

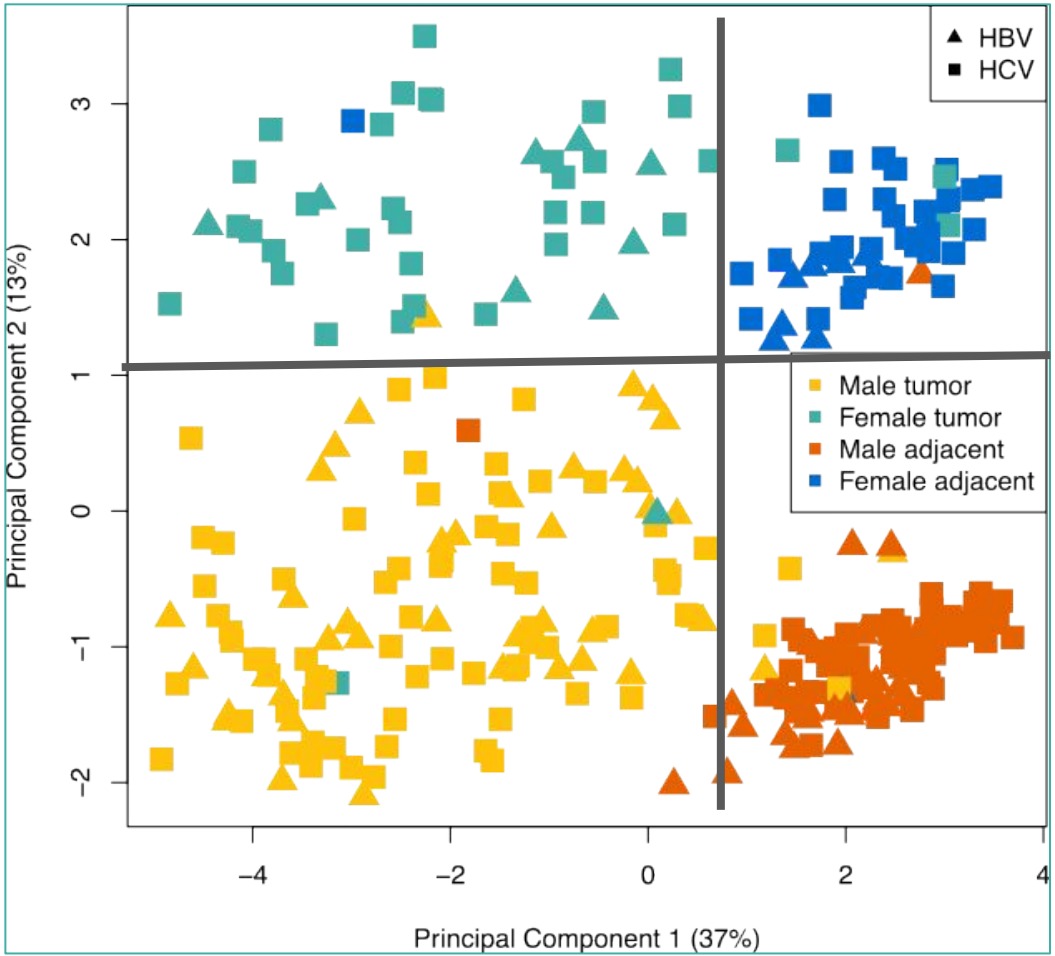
Tumor vs. Tumor - adjacent

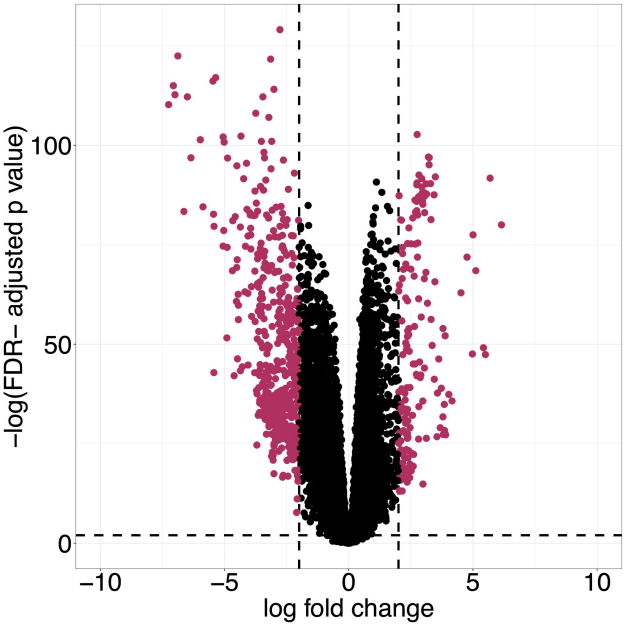
509 downregulated in tumors

158 upregulated in tumors



Sex drives differential gene expression in viral-mediated HCC





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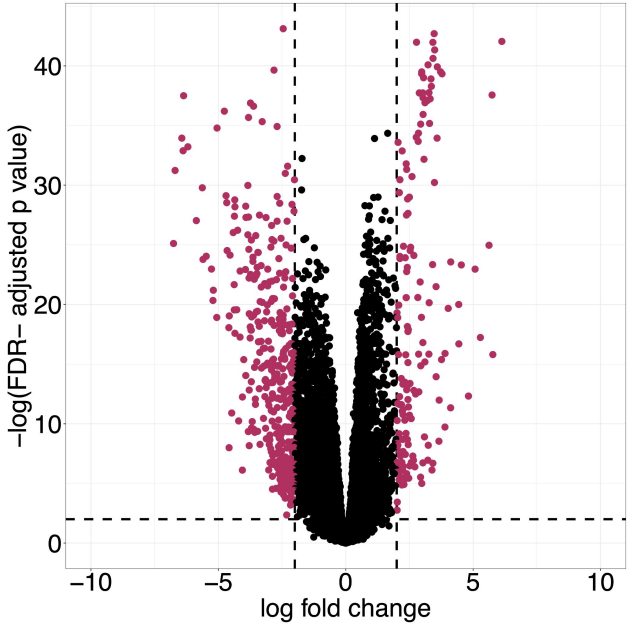
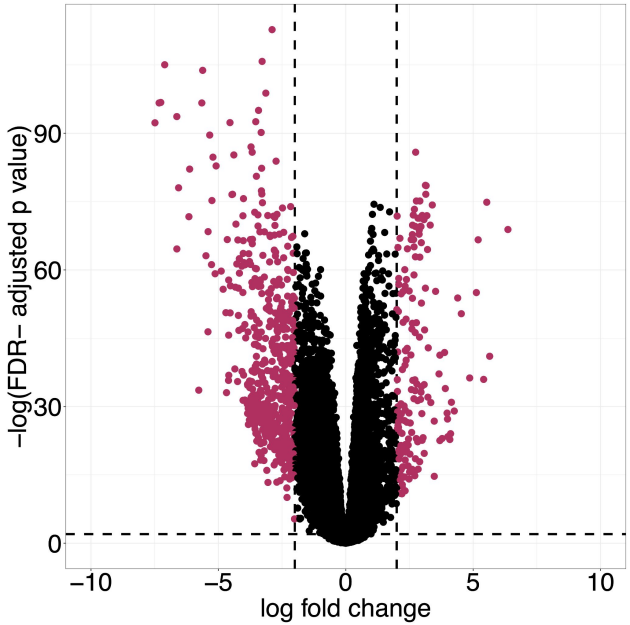


Female Tumor vs. Tumor - adjacent

430 downregulated

157 upregulated



Male Tumor vs. Tumor - adjacent

542 downregulated

164 upregulated

Differentially expressed genes→ logFC ≥ 2 and p.value < 0.05

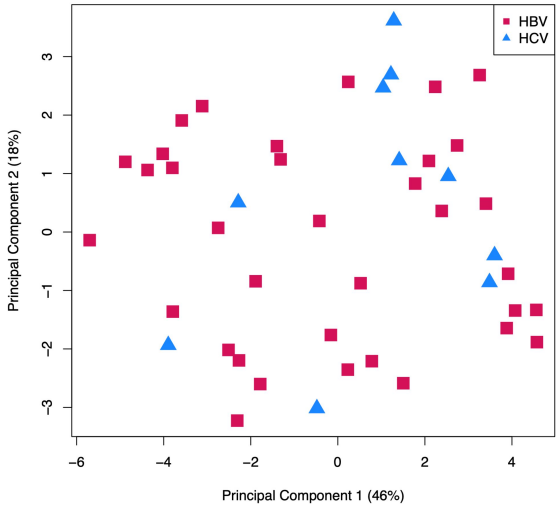
67.7% are shared between males and females, however 65 (8.4%) genes unique to female 184 genes (23.9%) unique in male.

H19 is upregulated in male tumor vs. tumor-adjacent but not female tumor vs. tumor-adjacent comparisons

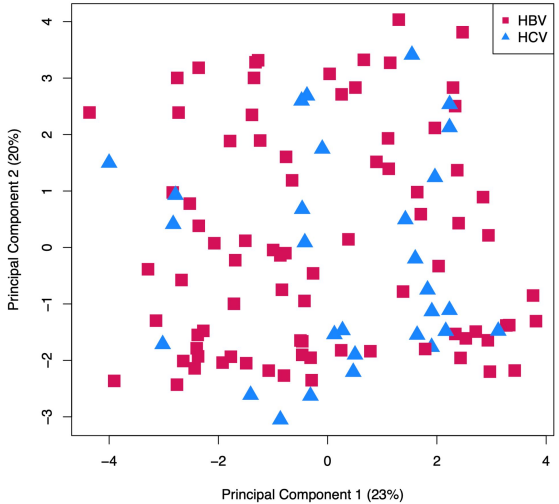
- Oncogenic long coding RNA
- H19 is paternal imprinted and maternally expressed
  - Paternal copy silenced only maternal copy active
- H19 is expressed in embryo → Repressed at birth → Re-expressed in some cancers
- Found as potential oncogene in HCC in mouse models

eEF1α2 downregulated in male tumor vs. tumor-adjacent but not female tumor vs. tumor-adjacent comparisons

- Located on the 20th chromosome
- Encodes two isoforms of alpha subunit of elongation factor-1 complex
  - Alpha 1 expressed in liver
- Found as potential oncogene in HCC in mouse models

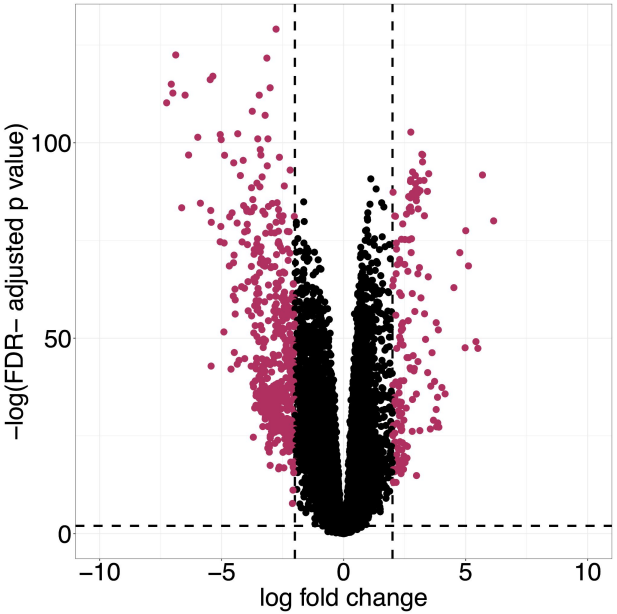


Female Tumor



Male Tumor

Multidimensional scaling analysis on male and female tumor tumor-adjacent samples does not show strong effect of etiology on sample variation



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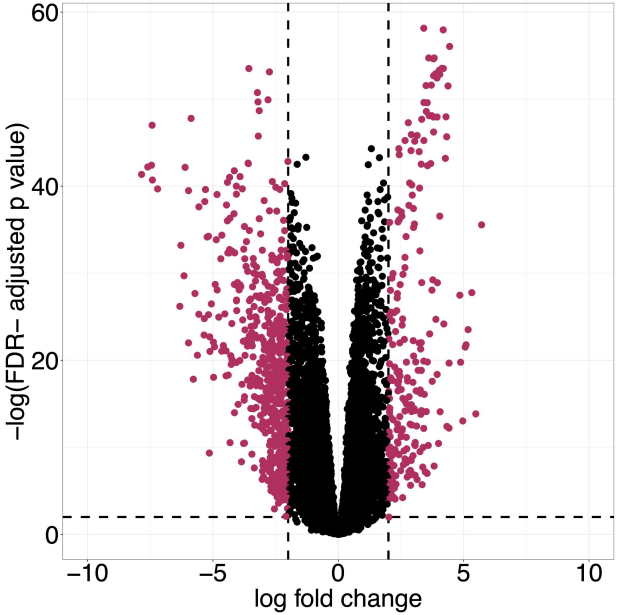
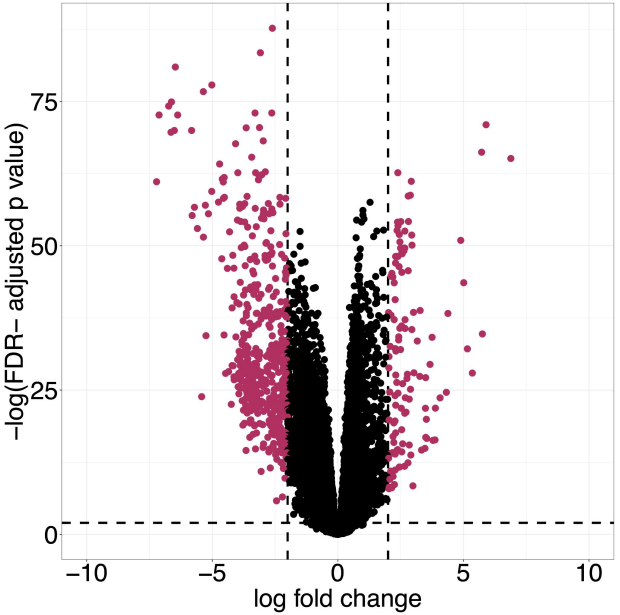


HBV Tumor vs. Tumor - adjacent

586 downregulated

238 upregulated



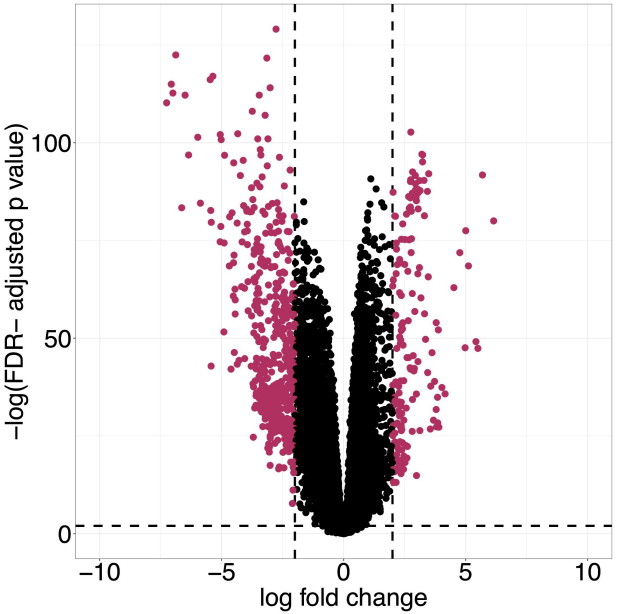
HCV Tumor vs. Tumor - adjacent

514 downregulated

124 upregulated

Differentially expressed genes→ logFC ≥ 2 and p.value < 0.05

43.5% of genes are shared between HBV and HCV tumor tumor-adjacent comparisons, however 381 (37.4%) genes unique to HBV 195 (19.1%) genes unique to HCV



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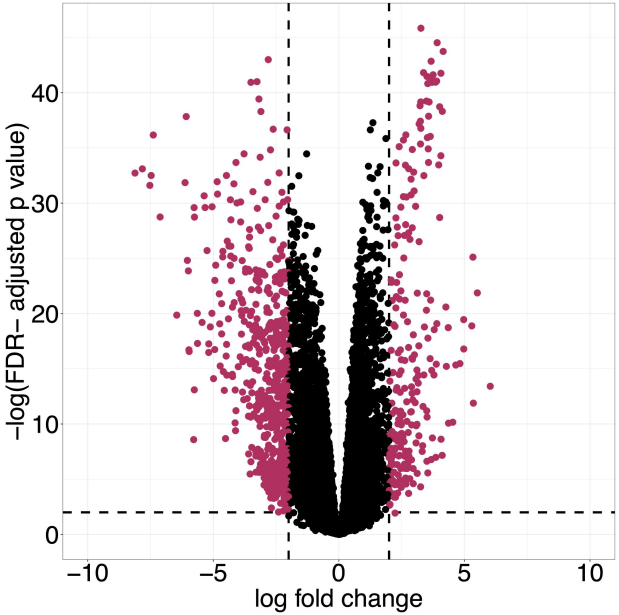
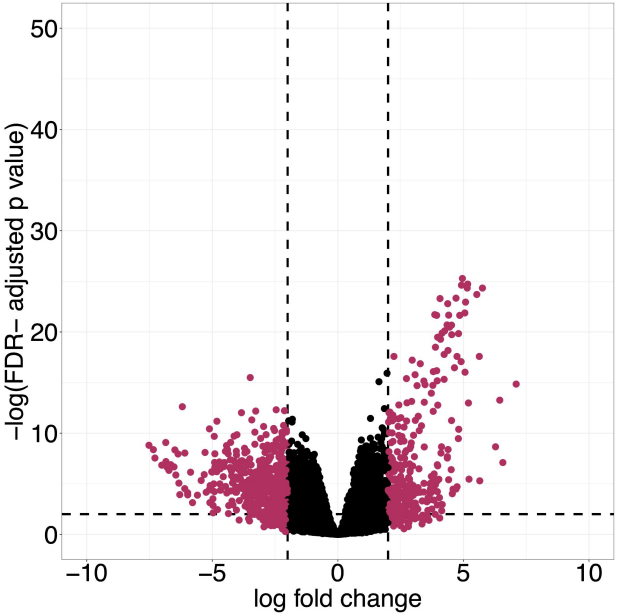


Male HBV Tumor vs. Tumor - adjacent

586 downregulated

238 upregulated



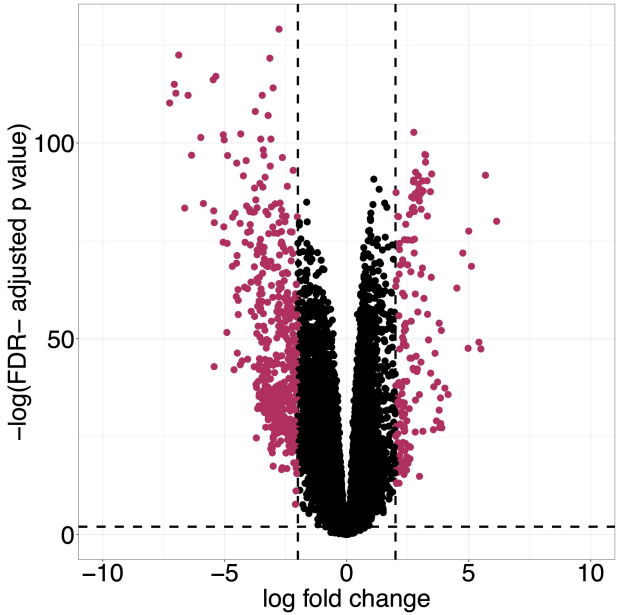
Female HBV Tumor vs. Tumor - adjacent

543 downregulated

305 upregulated

Differentially expressed genes→ logFC ≥ 2 and p.value < 0.05

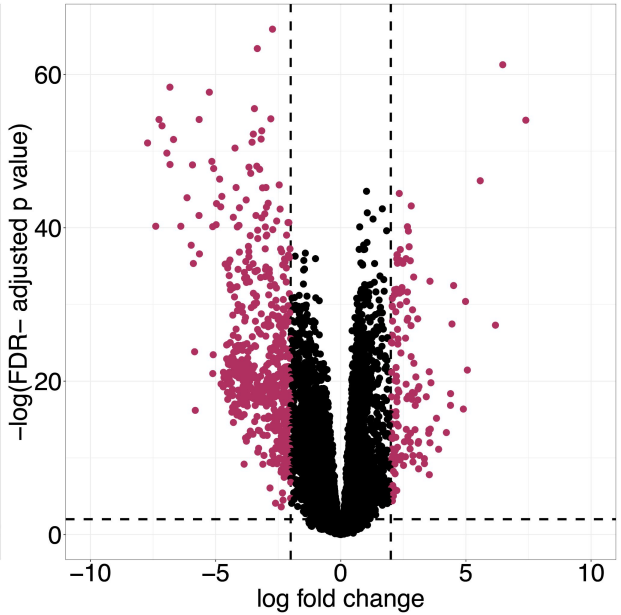
48.8% of genes are shared between male and female HBV tumor tumor-adjacent comparisons, however, 290 (25.5%) genes unique to male HBV, and 292 (25.7%) genes unique to female HBV



Tumor vs. Tumor - adjacent

509 downregulated in tumors

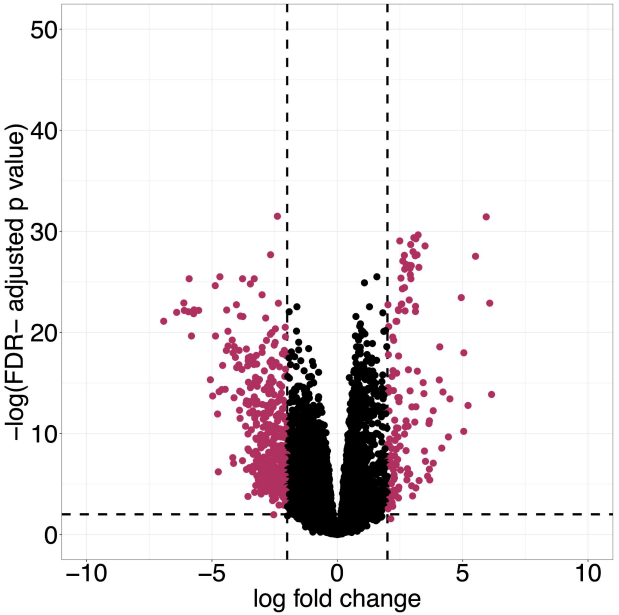
158 upregulated in tumors



Male HCV Tumor vs. Tumor - adjacent

578 downregulated

140 upregulated



Female HCV Tumor vs. Tumor - adjacent

435 downregulated

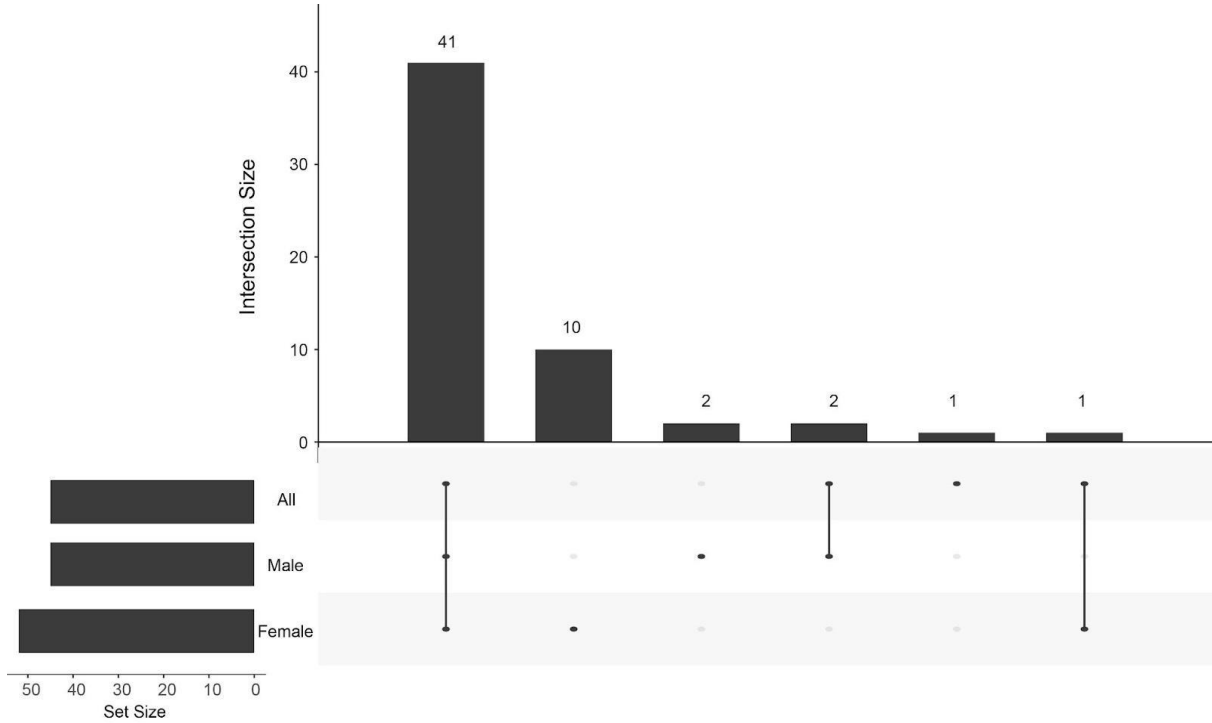
136 upregulated

Differentially expressed genes→ logFC ≥ 2 and p.value < 0.05

59.5% of genes are shared between male and female HCV tumor tumor-adjacent comparisons, however, 237 (29.3%) genes unique to male HCV, and 90 (11.1%) genes unique to female HCV

Pathway analysis reveals enrichment of cell cycle pathways in female differentially expressed genes and immune pathways in male differentially expressed genes

- Reactome pathway analysis
- Enriched pathways p-value < 0.05



Genes differentially expressed between female tumor vs. tumor-adjacent samples are enriched for involvement in cell cycle pathways

- Ten pathways enriched in females
- Four associated with the mitotic cell cycle
  - Cell cycle Mitotic
  - Phosphorylation and Emi1
  - Activation of NIMA Kinases NEK9, NEK6, NEK7
  - Resolution of Sister Chromatid Cohesion
- Mitotic cell cycle pathways have been shown to be enriched in HCC

Cell Cycle			
Cell Cycle Mitotic			
Regulation of mitotic cell cycle*	APC/C-mediated degradation of cell cycle proteins*	Regulation of APC/C activators between G1/S and early anaphase*	Phosphorylation and Emi1
M Phase*	Mitotic Prophase*	Nuclear Envelope Breakdown*	Activation of NIMA Kinases NEK9, NEK6, NEK7
	Mitotic Prometaphase*		Resolution of Sister Chromatid Cohesion
Drug ADME*			
Aspirin ADME			
Signal Transduction*			
Signaling by GPCR*			
GPCR ligand binding*	Class A/1 Rhodopsin-like receptor*	Amine ligand-binding receptor*	Adrenoceptors
Metabolism*			
Metabolism of ligands*			
Fatty acid metabolism*	Arachidonic acid metabolism*	Synthesis of (16-20)-hydroxyeicosatetraenoic acids (HETE)	
Biological Oxidations*			
Phase I - Functionalization of compounds*	Cytochrome P450 - arranged by substrate type*	Xenobiotics	CYP2E1 reactions

* Added for grouping purposes not significant

Genes differentially expressed between male tumor vs. tumor-adjacent are enriched for involvement with the immune system

- Two Cytokine Signaling pathways enriched
  - Interleukin-33 signaling
  - Interleukin-10 (IL-10) signaling
- Cytokines closely associated with HCC
- Studies have linked high IL-10 protein levels with lower survival rates

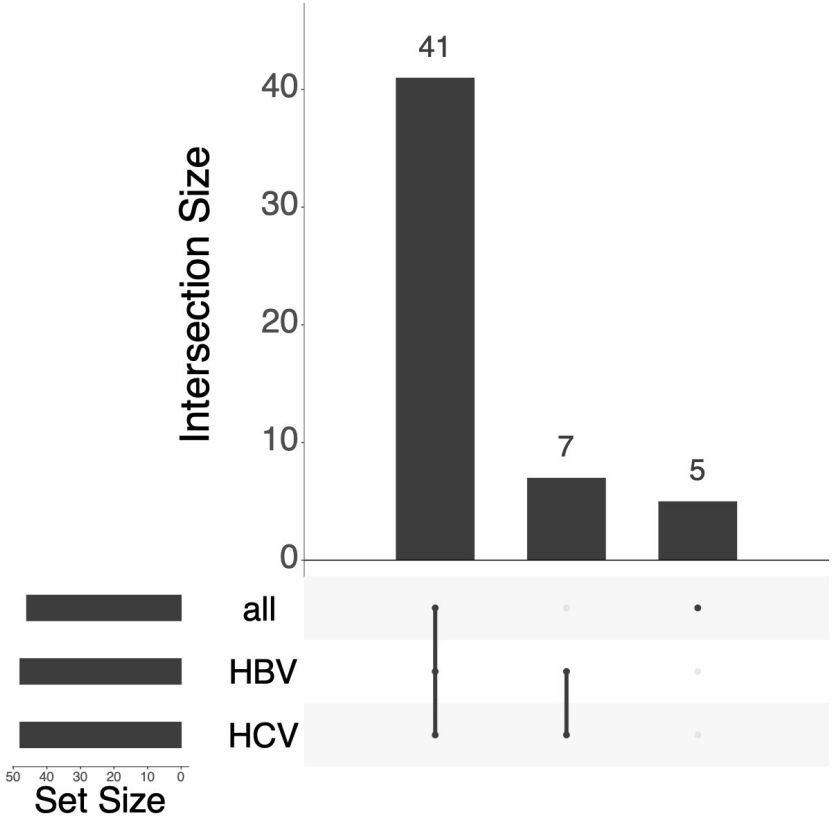
Transport of small molecules*		
Plasma lipoprotein assembly, remodeling, and clearance*		
Plasma lipoprotein remodeling		
Immune System*		
Cytokine Signaling in Immune system		
Signaling by Interleukins*	Interleukin-1 family signaling*	Interleukin-33 signaling
	Interleukin-10 signaling**	
Innate Immune System		
Complement Cascade*	Terminal pathway of complement**	

* Added for grouping purposes not significant

** Pathways shared by overall tumor tumor-adjacent genes and male tumor tumor-adjacent genes

Differentially expressed genes in HBV and HCV tumor vs. tumor-adjacent have no pathways unique to a specific etiology.

- Reactome pathway analysis
- Enriched pathways p-value < 0.05



Pathways enriched in shared genes have know associations with HCC and HCV

- Seven pathways shared with HCV and HBV
  - Drug ADME
  - Aspirin ADME
  - **Synthesis of (16-20)-hydroxyeicosatetraenoic acids (HETE)**
  - Cell Cycle, Mitotic
  - **Phosphorylation and Emi1**
  - G2/M DNA replication checkpoints
  - Plasma lipoprotein remodeling
- HCV stimulated G1/S pathway which activates CDKs
  - Phosphorylation and Emi1 is activated by CDKs
- HCV dysregulates host lipid metabolism
  - Synthesis of (16-20)-hydroxyeicosatetraenoic acids (HETE) associated with lipid metabolism

Drug ADME			
Aspirin ADME			
Metabolism*			
Metabolism of lipids*			
Fatty acid metabolism*	Arachidonic acid metabolism*	Synthesis of (16-20)-hydroxyeicosatetraenoic acids (HETE)	
Cell Cycle*			
Cell Cycle, Mitotic			
Regulation of mitotic cell cycle*	APC/C-mediated degradation of cell cycle proteins*	Regulation of APC/C activators between G1/S and early anaphase*	Phosphorylation and Emi1
Cell Cycle Checkpoints*			
G2/M Checkpoints*	G2/M DNA replication checkpoints		
Transport of small molecules*			
Plasma lipoprotein assembly, remodeling, and clearance*			
Plasma lipoprotein remodeling			

* Added for grouping purposes not significant

Sex-biased differentially expressed genes and enriched pathways are potential targets for individualized medicine

- We identified differentially expressed genes and enriched pathways that are sex-specific
- We identified differentially expressed genes and enriched pathways that are viral etiology specific
- These results will help create individualized diagnostics and therapeutics for viral-mediated HCC

Acknowledgements

- **Members of Dr. Wilson’s Sex Chromosome Lab**
- **Thesis Committee**
  - Dr. Kenneth Buetow
  - Dr. Melissa Wilson
  - Elizabeth Borden
- **My family and friends**

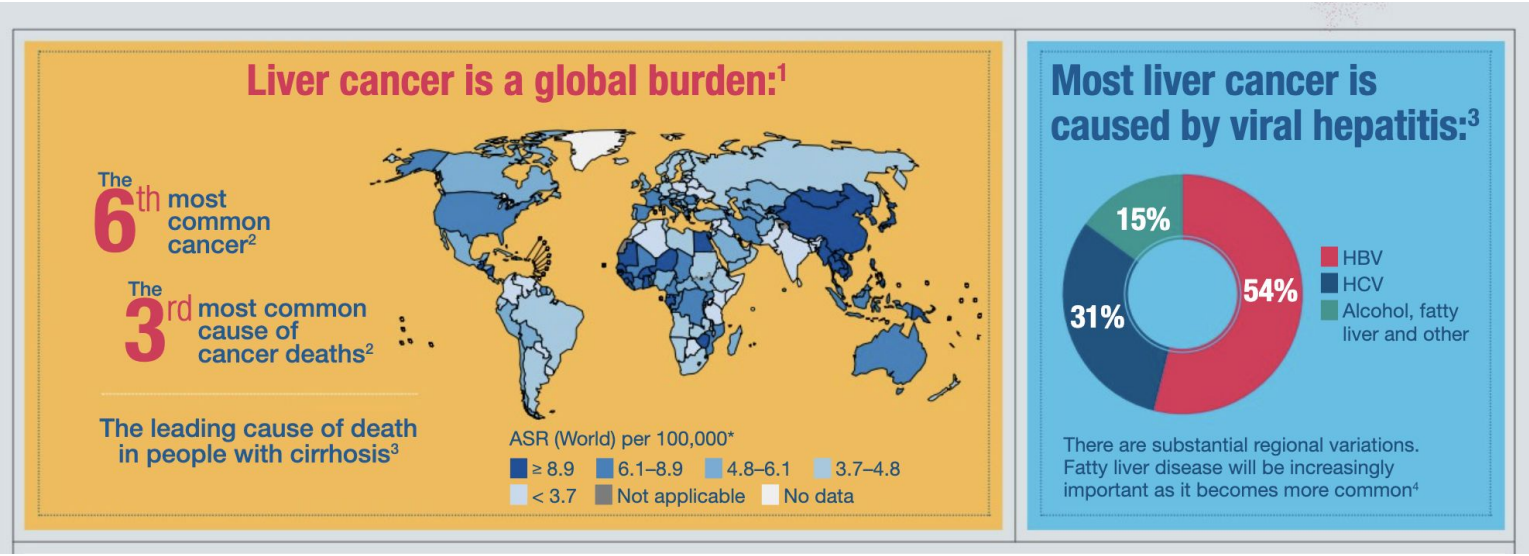
Questions?

The number of unique genes show the possibility significant effect of sex and etiology on sample variation

- The number of unique genes in each viral etiology suggest the possibility of significant differentiated genes that are etiology specific
- Further investigation is needed

Viral-mediated Hepatocellular Carcinoma poses a considerable health problem worldwide

- Liver cancer in the second deadliest cancer worldwide [4]
- Hepatocellular Carcinoma (HCC) accounts for 75% of all cases [1]
- Hepatocellular Carcinoma (HCC) is increasing prevalence in many countries [16]
- 80% of cases are mediated by Hepatitis B virus and Hepatitis C virus [8]



*Taken from Liver Cancer Explained | EASL Campus

MDS analysis reveals that etiology does not have a strong effect on sample variation

- The MDS analysis found that etiology does not have a strong effect on sample variation
- The number of unique genes in each viral etiology suggest the possibility of significant differentiated genes that are etiology specific
- The number unique genes contradicts the results from the MDS plot
- Further investigation needed

What We Don’t Know

- We aim to discover distinct gene regulatory functions between sexes and viral etiologies.

- This will help provide an understanding of biological mechanisms underlying sex and viral etiological differences in viral-mediated HCC.

- This understanding will aid in the creation of sex-specific cancer diagnostics and therapeutics

The goal of the differential expression analysis is to see for a given gene if the observed expression difference between tumor and tumor-adjacent is greater than what would be expected due to random variation.

The goal of pathway enrichment analysis is to see if any gene regulatory networks are overrepresented in a groups of genes than what would be expected by random chance.

Sex drives differential gene expression in viral-mediated hepatocellular carcinoma

- Sex drives differential gene expression
  - Separation of samples on MDS analysis on overall tumor tumor-adjacent pairwise comparison is driven by tumor vs. tumor-adjacent and sex (Figure 1)
  - List number of genes upregulated and downregulated genes in tumor (Figure 2-4)
  - Majority of DEGs shared between male and female but 184 number are unique to male and 65 are unique to female - calculate percentage unique vs. shared

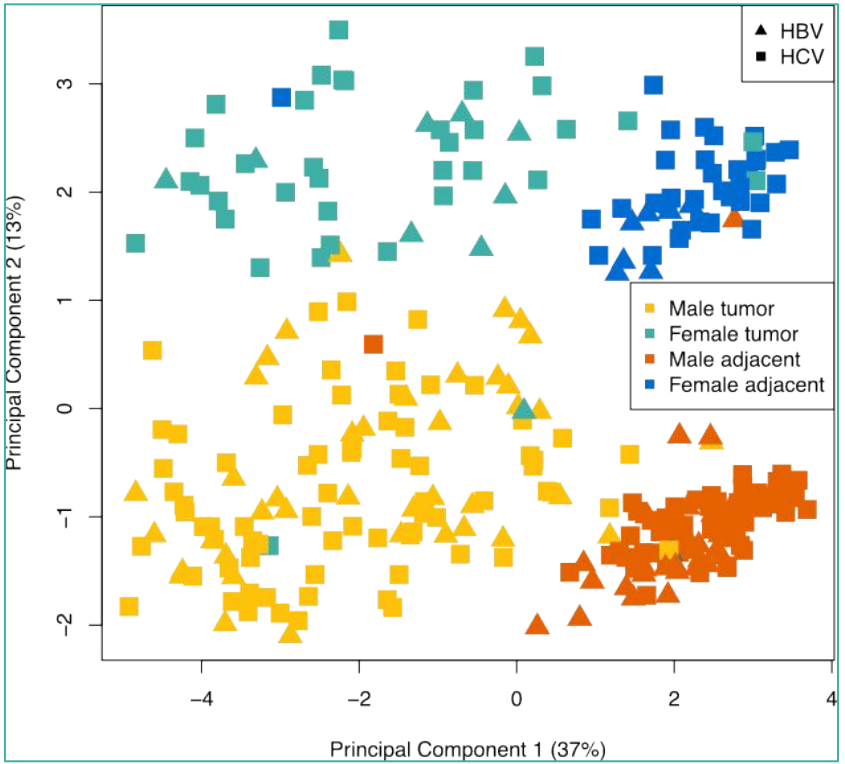
Goal: Determine sex differences in differentially expressed genes and pathways in viral mediated liver cancer

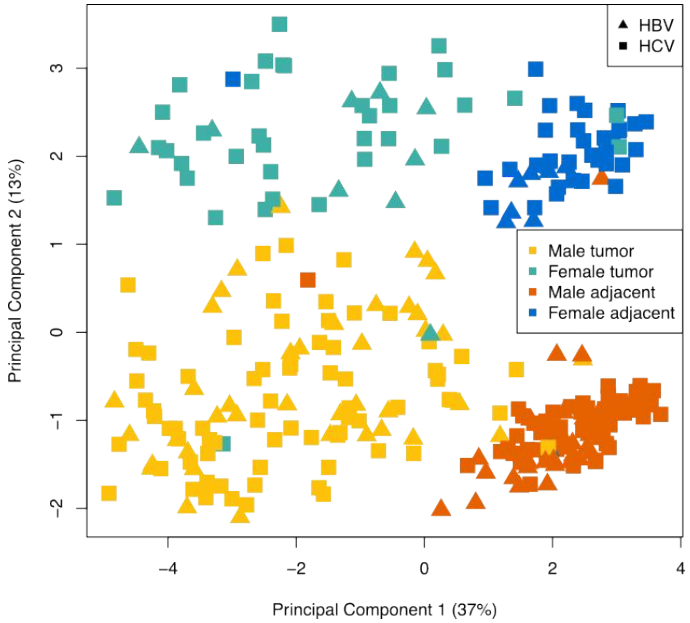
1. Identify differentially expressed genes subsetted by sex, etiology, or both
2. Identify enriched pathways in genes subsetted by sex, etiology, or both

Sex drives differential gene expression in viral-mediated HCC

Multidimensional scaling analysis on top 50 genes

Demonstrates impact of sex on explaining the variation in the samples

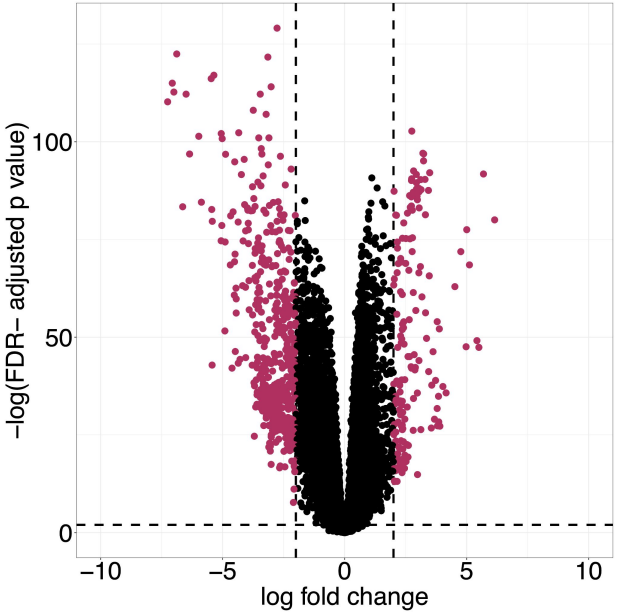




MDS plot

Principal component 1 37% -- attributable tumor vs. tumor-adjacent

Principal component 2 12%-- male vs. female sex



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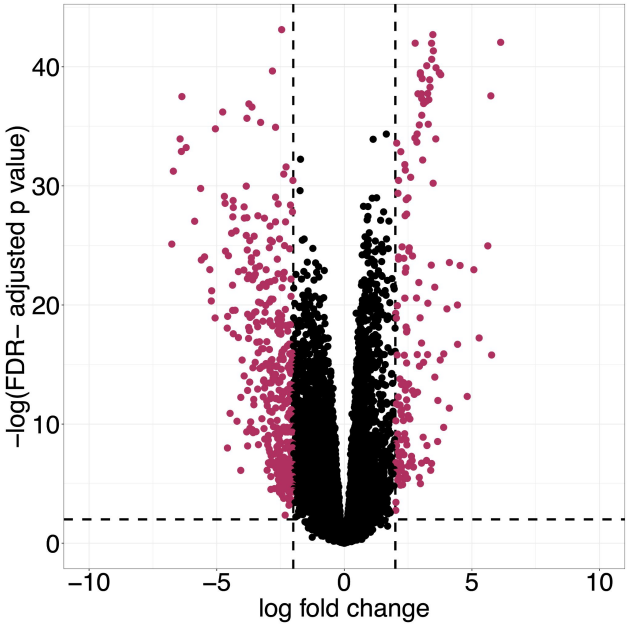
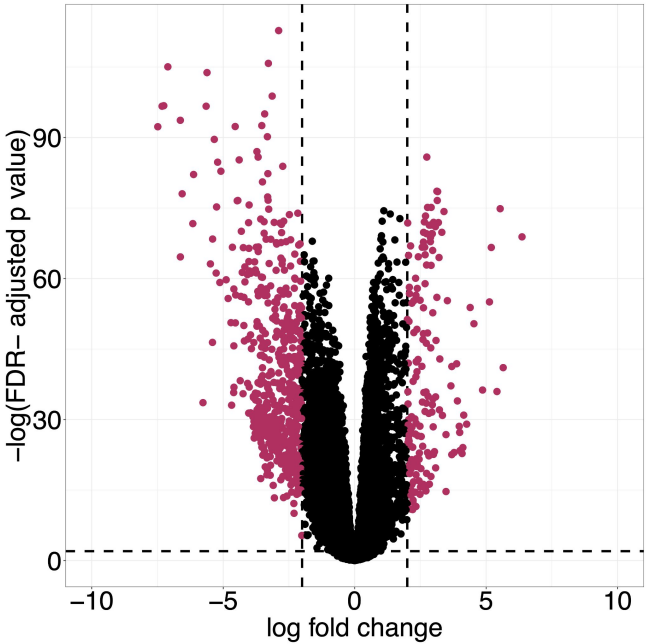


Female Tumor vs. Tumor - adjacent

430 downregulated

157 upregulated



Male Tumor vs. Tumor - adjacent

542 downregulated

164 upregulated

67.7% are shared between males and females, however 65 (8.4%) genes unique to female
184 genes (23.9%) unique in male.

Multidimensional scaling analysis does not reveal a strong effect of etiology on sample variation differential gene expression.

- Multidimensional scaling analysis on male and female tumor tumor- adjacent samples does not show strong effect of etiology on sample variation(Figure 5)
- Majority of DEGs shared between HBV and HCV with X and Y Figure (6-7)

Majority of differentially expressed genes separated by both sex and etiology are shared by both males and females.

- List number of genes upregulated and downregulated in tumor (Figures 9-12)
- Number of DEGs shared between male and female HBV (Figure 9,11).
- If number of DEGs is small mention that if it is large talk about further investigation is require
- Number of DEGs shared between male and female HCV (Figures 10,12)
- If number of DEGs is small about it if it is large further investigation is required

Genes differentially expressed between female tumor vs. tumor-adjacent samples are enriched for involvement in cell cycle pathways

- Majority of enriched pathways are shared between male and female differentially expressed genes (Figure 13)
- Ten pathways are enriched (Table 3)
- Pathways enriched are associated with the cell cycle

Genes differentially expressed between male tumor vs. tumor-adjacent are enriched for involvement with the immune system.

- Small number (2) of pathways are enriched (Table 4)
- Pathways enriched associated with immune system

Goal: Determine sex differences in differentially expressed genes and pathways in viral mediated liver cancer

1. Identify differentially expressed genes subsetted by sex, etiology, or both
2. Identify enriched pathways in genes subsetted by sex, etiology, or both

Differentially expressed genes in Hepatitis B tumor vs. tumor-adjacent tissue and Hepatitis C tumor vs. tumor-adjacent tissue have no pathways unique to a specific etiology.

- No pathways unique to HBV and HCV
- Seven pathways enriched in both HBV and HCV (Table
- 5 pathways unique to overall tumor tumor adjacent and require further investigation

Drivers of genetic gene expression→ Sex

- We found gene dysregulation in tumor that is consistent with our understanding of carcinogenesis.
- We found a number of genes to be sex differentially expressed which is consistent with the idea that HCC is a sex biased cancer
    - Consistent after relaxing p. Value for females from 0.05→0.1
-

Conclusions

- Identified sex-biased differentially expressed genes
- Identified differentially expressed genes specific to viral-etiologies
- Identified potential pathways enriched by sex specific differentially expressed genes
- Identified potential pathways enriched by viral-etiology specific differentially expressed genes

Conclusions

- Distinct regulatory functions between sexes and viral etiologies
- Created a framework for discovering sex-biased genetic expression and regulatory networks in viral mediated sex-biased cancers
- Created a framework for discovering viral-etiology genetic expression and regulatory network in viral mediated sex-biased cancers

Identified sex-biased differentially expressed genes

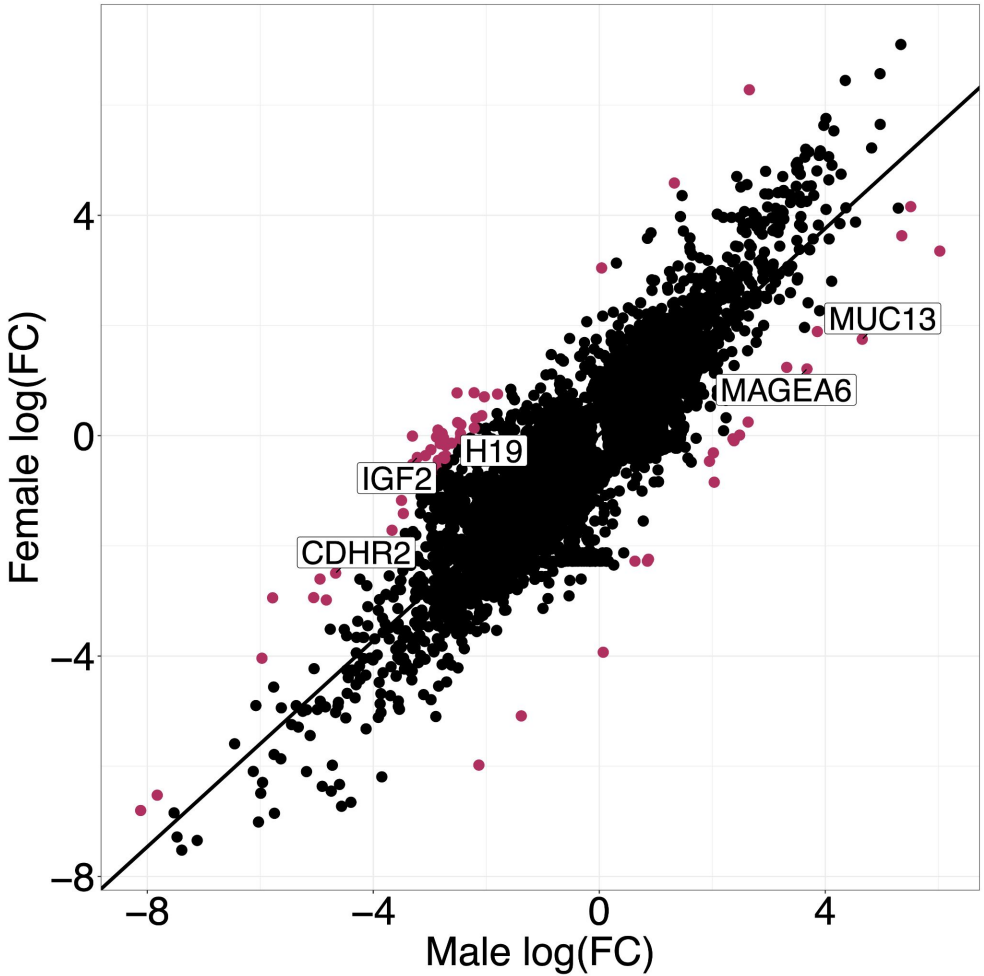
Identified DEGs specific to viral-etiologies

Identified potential pathways… sex-biased viral-etogies

Supplementary Slides

HBV logFC plot

- Maroon dots indicate residual > 2.25



Further Investigations

- Identify enriched pathways that are sex-biased and viral etiology specific
- Further investigate how etiology drives differential gene expression

Supplementary Slides→ Methods

- Trimmomatic parameters
  - 2 seed mismatches, palindrome clip threshold 30, simple clip threshold 10, leading quality value 3, trailing quality value 3, sliding window size 4, minimum window quality 30, and minimum read length of 50
- Why sex-specific?
  - To overcome mismapping of short sequencing reads due to sequence homology on X and Y chromosomes the reads were mapped to a sex-specific reference genome.
- sample ID "RK023" was removed from the dataset due to low quality.

Methods→ Samples, Alignment Quantification, Filtering

- FASTA→FASTQC [2] quality control→trimmed using Trimmomatic [3] → Hisat2 mapping to GRCh38.p7 sex-specific genome [11] → Quantified by *Subread featureCounts* [15]
- Genes were retained based on FPKM value of ≥ 0.5 and read count of > 6 in at least 10 samples
  - FPKM → fragments per kilobase of exon per million fragments mapped
  - TMM → Trimmed Mean of M-values

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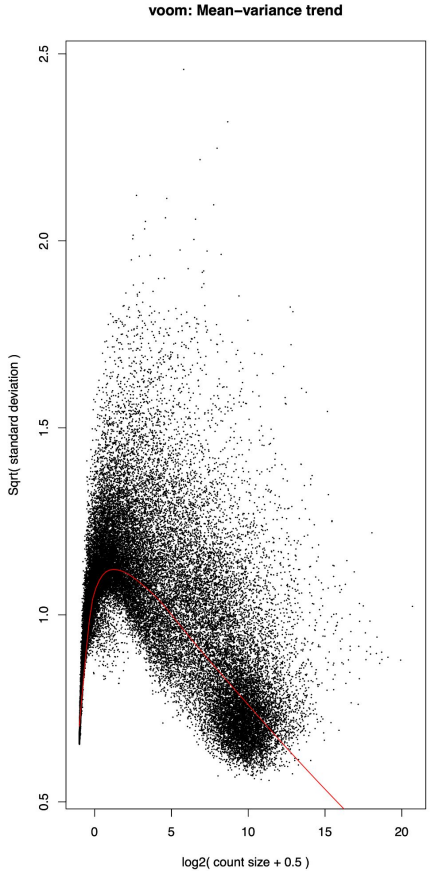
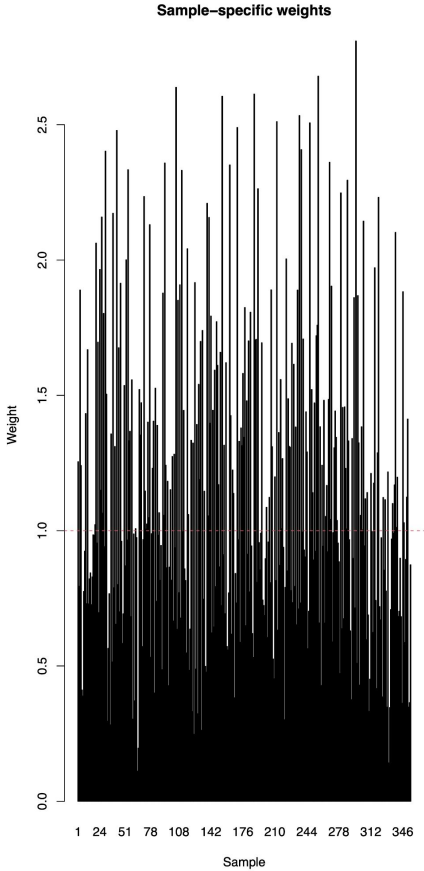
HBV	33	40	8	9
HCV	59	72	34	36
HBV & HCV	4	4	0	0
Total	**121**	**140**	**8**	**9**

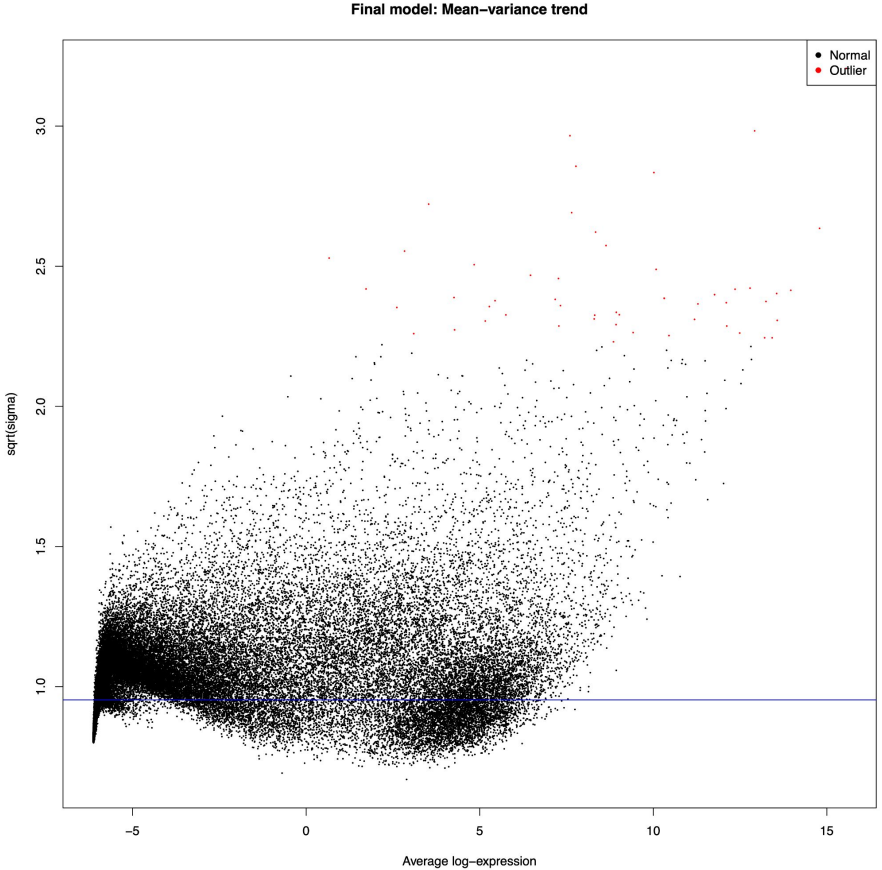
Sample distribution

Methods→ Differential Expression

- Design matrix had lesion type as predictor variable
  - Library added as covariate
  - Sex added as covariate to overall tumor vs. tumor-adjacent and tumor tumor-adjacent stratified by viral etiology
- *voomWithQualityWeights log2 normalized* adjusted raw reads for quality [14]
  - Weights were passed into limma pipeline
- *duplicateCorrelation* function computed correlation between tumor tumor-adjacent samples on the same patient [20]
  - Included in limma pipeline
- Limma generates linear model using the voom weights and correlation values
- Empirical bayes smoothing increased the power of the analysis


voom: Mean−variance trend
Sqrt(standard deviation)
log2(count size + 0.5)
0.5
1.0
1.5
2.0
2.5
0
5
10
15
20


Sample−specific weights
Weight
Sample
0.0
0.5
1.0
1.5
2.0
2.5
1
24
51
78
108
142
176
210
244
278
312
346


Final model: Mean−variance trend
sqrt(sigma)
Average log−expression
Normal
Outlier
1.0
1.5
2.0
2.5
3.0
−5
0
5
10
15

Methods → Differential Expression, Pathway Enrichment

- **Genes were assumed to be differentially expressed if there was a log fold change (logFC) ≥ 2 and p value of < 0.05**
- Differentially expressed genes were compiled and analyzed by Reactome using over-representation analysis [9]
- **Pathways were considered enriched if they had p-value of less than 0.05.**

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