Pathway Analysis Reveals Sex Differences in Human Hepatocellular Carcinoma

by

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ABSTRACT

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide and exhibits a male-bias in occurrence and mortality. Previous studies have provided insight into the role of inherited genetic regulation of transcription in modulating sex-differences in HCC etiology and mortality. This study uses pathway analysis to add insight into the biological processes that drive sex-differences in HCC etiology as well as a provide additional framework for future studies on sex-biased cancers. Gene expression data from normal, tumor adjacent, and HCC liver tissue were used to calculate pathway scores using a tool called PathOlogist that not only takes into consideration the molecules in a biological pathway, but also the interaction type and directionality of the signaling pathways. Analysis of the pathway scores uncovered etiologically relevant pathways differentiating male and female HCC. In normal and tumor adjacent liver tissue, males showed higher activity of pathways related to translation factors and signaling. Females did not show higher activity of any pathways compared to males in normal and tumor adjacent liver tissue. Work suggest biologic processes that underlie sex-biases in HCC occurrence and mortality. Both males and females differed in the activation of pathways related apoptosis, cell cycle, signaling, and metabolism in HCC. These results identify clinically relevant pathways for future research and therapeutic targeting.

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INTRODUCTION

1. Biological differences between sexes in cancer

Sex-biases in cancer occurrence and mortality are evident across multiple histological subtypes. For example, the mortality of cancer has been shown to be greater in males than in females, with the leading causes of cancer deaths: lung, colorectal and stomach cancers showing higher mortality in men (Siegel et al. 2020; Fitzmaurice et al. 2017). Additionally, males show poorer response to treatments, including some forms of chemotherapy and immunotherapy (Clocchiatti et al. 2016). Differences in risk factors and life-styles account for a portion of the sex bias, but adjustment of these factors shows that there are other biological differences (Ouyang et al 2015; Wisnivesky and Halm 2007). The circulation of sex hormones has been shown to contribute to the genesis and progression of some cancers. Breast and prostate cancer respond strongly to circulating sex hormones. Additionally, estrogen has been shown to be anti-tumorigenic for liver and colon cancers. However, the molecular differences for the sex disparity in most cancer is still undefined. To identify the sex-specific molecular differences, one group examined the mutational profiles of tumors from both males and females across The Cancer Genome Atlas (TCGA) (Li et al. 2018). They found that different genes were associated with tumor aggression in each sex. This called for increased study and consideration of the molecular role of sex in cancer etiology, progression, treatment, and personalized therapy. Still, despite the clear molecular differences underlying these characteristics in females and males, sex is rarely considered as a variable in clinical research.

2. Sex-biased occurrence in hepatocellular carcinoma

Of the tumor types analyzed by Li et al. 2018, the largest sex differences in autosomal mutational profiles were identified in hepatocellular carcinoma (HCC). HCC exhibits a male-bias in occurrence, with a male-to-female incidence ratio between 1.3:1 and 5.5:1 across populations (Wands 2007; Petrick et al 2016). Furthermore, males exhibit a different manifestation of HCC with earlier onset and more/larger nodules in clinical studies. Across the United States, HCC is the fifth leading cause of cancer deaths and is one of the few cancers that is increasing in both incidence and death (Siegal et al. 2020). Factors such as metabolic conditions, fat-related liver disease and cirrhosis have become increasingly prominent in recent decades. These factors have been shown to have influence on the sex-biases observed in HCC. Men are two times more likely to die from chronic liver disease and cirrhosis (Guy and Peters 2013). However, very little research has been performed that examines the etiological origins of the liver diseases and how they all relate to sex-biases within HCC. With HCC becoming a leading cause of cancer incidence and death, it is clear that more research into the development of therapeutic treatments is needed.

3. Previous research on sex differences in hepatocellular carcinoma

Sex-specific regulation of genes may partially drive sex differences in risk and severity. A previous study observed extensive sex-biased signatures in gene expression in HCC and other cancers that show sex-biased occurrence and mortality (Yuan et al. 2016). However, this study focused on female and male tumor profiles without consideration of normal or tumor adjacent tissue. It is necessary to contrast the sex differences in the gene

expression of tumor tissue with those in normal or tumor adjacent tissue to fully understand cancer-specific processes. Targeted treatment uses this combined understanding to distinguish whether sex differences are a factor of inherent differences in healthy individuals or cancer-specific processes. Furthermore, the study of normal and tumor adjacent tissue is very important in the understanding of disease etiology and occurrence.

The complexity of HCC makes studying its etiology with single genes or mutations difficult. Therefore, many researchers have turned to pathway analysis to better understand this disease. Pathways are sets of molecular interactions that underlie a given function. Analysis at the pathway level allows one to capture the complexity of real biological processes. Central to pathway analysis is the idea that disruption of a pathway, not just a single gene component, could be the basis for diseases such as HCC. Recently, a study applied sex-stratified gene expression analysis to identify sex-specific molecular etiologies of HCC in normal, tumor adjacent, and tumor liver tissue (Natri et al. 2019). They used differential expression and eQTL analysis to identify etiologically relevant genes differentiating females and males. Then, to identify sex-shared and sex-specific pathways driving HCC etiology, they calculated the overrepresentation of genes in regard to a set of pathways. While this approach to pathway analysis is important to place a set of genes in its function, it does not consider a pathway's interactive components. Here, we used PathOlogist (https://github.com/Buetow-Lab/PathOlogist) to identify etiologically relevant pathways differentiating females and males.

4. Pathway analysis: PathOlogist and its benefits over traditional gene and gene-set analysis

PathOlogist uses RNA expression data to calculate two quantitative measurements: ‘activity’ and ‘consistency’ for each sample in a set of pathways. These metrics make use of the structure of gene interactions within the pathway, instead of treating the genes as a simple list of entities (Efroni et al. 2007). A pathway is defined as a network of molecular interactions. Each interaction consists of one or more input genes, promoters, and inhibitors, and one or more output genes. Activity and consistency scores are calculated for each interaction based on the expression of all input and output genes. Activity scores are a measurement of how likely the interactions are “on” (or active) while the consistency scores determine whether these interactions follow the logic of the defined pathway structure or has been “rewired.” These scores can reveal a variety of different information depending on the experiment. One may compare activity scores to determine if a functional process is differentially activated or inactivated between certain groups or in response to a treatment. Comparing consistency scores can reveal if a pathway’s structure has been altered by a disease, such as HCC. A study used activity as a metric to identify biomarkers that predict a cancer’s response to a drug (Ben-Hamo et al. 2020). They found that these metrics were more efficient than gene expression alone and helped reduce experimental platform effects.

Here we use PathOlogist to calculate activity and consistency scores from TCGA and The Genotype-Tissue Expression Project (GTEx). Then we use statistical analysis to identify sex-specific alterations in biological processes in within tissue and between

tissue analysis. We provide potential molecular mechanisms for the sex-biased occurrence and mortality in HCC. The results here have implications for the development of targeted treatments for female and male HCC.

METHODS

1. Data Acquisition

GTEx (release V6p) whole transcriptome (RNAseq) data (dbGaP accession: phs000424.v6.p1) was downloaded from dbGaP. TCGA LIHC RNAseq data was downloaded from NCI Genomic Data Commons (dbGaP accession: phs000178.v8.p7). In total, RNAseq data from 91 male and 44 female GTEx donors, and 253 male and 121 female TCGA LIHC donors, as well as paired tumor and tumor-adjacent samples from 28 male and 22 female TCGA LIHC donors were used in this study.

Raw fastq files were trimmed using *TRIMMOMATIC* (v0.33) (Bolger et al. 2014) with default parameters. Raw counts were generated by Salmon (v1.1.0) (Patro et al. 2017) against human genome version 2.9 from https://www.gencodegenes.org/ using default setting. Gene-level counts were quantified using the *tximport* package in R then transformed using the *limma/edgeR* package to perform voom transformation (Law et al. 2014). The transformed data is exported into a tab-delimited text file.

1290 biological networks were obtained from the Pathway Interaction Database (PID), BioCarta, KEGG, and Reactome pathway databases (Schaefer et al 2009; Nishimura 2001; Kanehisa et al 2017; Fabregat et al 2017). Pathways were subjectively filtered for biological relevance and large categorical networks were omitted.

2. Calculation of pathway metrics

To calculate pathway activity metrics, we used the PathOlogist tool (Greenblum et al. 2011), which translates gene expression levels (in the manner detailed below) to a metric that provides information about the interactions within a pathway. This pathway activity is provided per sample, for each of the included pathways.

To use gene expression, PathOlogist first calculates, for each gene in each sample, the probability that the gene is in an alternative expression state in that specific sample. The higher expressed state of the gene is called an "up" gene (or "on"), and a lower expressed gene is called "down" (or "off"). A probability for a specific gene to be in the "up" state is calculated using the distribution of the expression level of the gene across all samples.

Across samples, we assume that the collection of unexpressed and expressed instances are a mixture of two gamma distributions. For each gene, we use an expectation-maximization (EM) algorithm to iterate over the data. The EM algorithm finally provides us with the most likely parameters of the modeled distributions and with the mixture weights of the two distributions. Once the distributions are in place, we can calculate the probability of each gene in a sample to belong to one of the two distributions. That is, we obtain the probability of the gene to be in the up state. This probability is the (Up Down Probability) UDP measure that is used in the pathway score. Since a gene, in principle, could be in an "up" state or in a "down" state across all samples, we need to determine if the best fit is a single gamma distribution or a mixture

of two gamma distributions. To compare these two models, we use the Bayesian information criterion (BIC).

Once we have the set of gene probabilities for each gene in each sample, we continue to calculate the pathway activity metric, which is calculated for all 1290 pathways. PathOlogist treats the pathway as a network of interactions and assigns the network a score based on the expression levels of the interacting genes and on the quality of the interaction. The analysis also takes into consideration the specific type of interaction (inhibition or promotion). The activity of each interaction is calculated by multiplying the probability of the genes to be "active" (based on the UDP matrix). Then, the final pathway activity score is calculated by averaging all the interaction activity scores in the pathway. Consistency scores compare this activity potential with the presence of output molecules in an interaction, providing an account of deviations from the pathway logic. Like activity scores, the final pathway consistency score is calculated by averaging all the interaction consistency scores in the pathway.

The final outputs are two tab-delimited text files containing the activity and consistency scores for each sample and for all 1290 pathways.

2. Statistical Analysis

a. Within tissue analysis

i. Wilcoxon rank sum test

A nonparametric Wilcoxon rank sum test was performed to find pathways whose scores can be used to differentiate two classes of samples (eg. female v. male). For sex-based comparisons, tissues were analyzed separately in three groups: GTEx female vs GTEx male, TCGA tumor adjacent female v. TCGA tumor adjacent male, and TCGA tumor female v. TCGA tumor male.

For each pathway a two-sample ranksum test is performed to evaluate the null hypothesis that independent pathway scores from class A and class B are pulled from the same mean and distribution. Significant pathways are those for which the null hypothesis is highly unlikely, indicating that the pathway's activity or consistency is different in these two groups of samples. The result of the ranksum test is a p-value which is then Bonferroni-corrected by multiplying it by the number of pathways being analyzed (1290).

ii. Binary classification algorithm

To further investigate the differences in male and female HCC, the samples were then classified based on their placement within a mixed gaussian distribution. This analysis takes advantage of the pathway metrics given by PathOlogist to calculate the probability that a sample is either 'active' or 'inactive' and 'consistent' or 'inconsistent.' By applying two gaussian distributions over the pathway metrics, we can use an EM

algorithm to calculate the probability that the sample was in the ‘active’ or ‘consistent’ distribution (Figure 4). If the probability was >= 0.5, then the sample was ‘active’ or ‘consistent,’ otherwise it was ‘inactive’ or ‘inconsistent.’

iii. Chi-squared analysis

Labels obtained from binary classification were analyzed using a chi-squared test and a contingency table to evaluate pathways where the proportion of active or consistent samples were significantly different between males and females using Bonferroni corrected p-values <= 0.05. The chi-squared test comparing females and males was repeated for each tissue type: GTEx normal, TCGA tumor adjacent, and TCGA tumor. We identified pathways that had differences in the proportional frequencies of active or consistent male and female samples.

b. Between tissue analysis

i. Chi-squared analysis

Binary classification data for each tissue type, normal tumor adjacent, and tumor was then used for a second set of between tissue analyses. Chi-squared analysis was performed for 9 comparisons in total, 3 sex-combined and 6 sex-specific between each tissue type. We identified pathways that had differences in the proportional frequencies of ‘active’ or ‘consistent’ samples between normal, tumor adjacent, and tumor tissues based on a Bonferroni adjusted p-value < 0.05.

ii. Log-linear modeling

Log-linear modeling was performed using the *vcd* package v1.4-8. Binary classification data from between tissue analysis was used along a list of labels designating the sample's sex and tissue type (eg. normal or tumor). A log-linear model utilizing a joint independence hypothesis was used to evaluate the association between a sample's state (eg. active or inactive) and sex while adjusting for its tissue type. It was repeated for 3 comparisons: GTEx all v. TCGA tumor adjacent all, GTEx all v. TCGA tumor all, and TCGA tumor adjacent all v. TCGA tumor all. We identified pathways where sex and state were associated while adjusting for tissue based on a Bonferroni adjusted Pearson's p-value <= 0.05.

TABLES/FIGURES

Table 1. *Significant pathways from Wilcoxon rank sum test in GTEx female v. GTEx male comparison.* Activity and consistency values were calculated using PathOlogist, then a Wilcoxon rank sum test was performed comparing male and female samples in GTEx normal liver tissue. A p-value threshold of <=0.05 was used to determine significance.

score type	pathway	p-value	male mean	female mean
activity	long-term potentiation(kegg)	7.23E-07	0.640156044	0.5706045455
activity	olfactory transduction(kegg)	3.71E-06	0.3173164835	0.2357295455
activity	vibrio cholerae infection(kegg)	4.74E-08	0.4199648352	0.2772136364
activity	wnt signaling pathway(kegg)	4.28E-10	0.3973131868	0.3468840909
activity	eukaryotic translation elongation(reactome)	0.00711	0.5598351648	0.5556
consistency	gap junction(kegg)	2.45E-06	0.6516318681	0.6993272727
consistency	long-term potentiation(kegg)	5.33E-16	0.7224296703	0.6290613636
consistency	wnt signaling pathway(kegg)	1.96E-08	0.5692395604	0.534825
consistency	selenoamino acid metabolism(reactome)	0.04952	0.8885659341	0.8940045455
consistency	eukaryotic translation elongation(reactome)	9.93E-08	0.8188340659	0.8426
consistency	translation(reactome)	0.02957	0.6819494505	0.6936545455
consistency	protein-protein interactions at synapses(reactome)	3.25E-07	0.7446483516	0.7915704545

Table 2. *Significant pathways from Wilcoxon rank sum test in TCGA tumor adjacent female v. TCGA tumor adjacent male comparison.* Activity and consistency values were calculated using PathOlogist, then a Wilcoxon rank sum test was performed comparing male and female samples in TCGA tumor adjacent liver tissue. A p-value threshold of <=0.05 was used to determine significance.

score type	pathway	p-value	male mean	female mean
activity	calcium signaling pathway(kegg)	0.00982	0.27982	0.21036
activity	long-term potentiation(kegg)	8.32E-06	0.60251	0.4886
activity	vibrio cholerae infection(kegg)	2.26E-06	0.91391	0.67845
consistency	gap junction(kegg)	0.04807	0.67818	0.71273
consistency	long-term potentiation(kegg)	1.32E-05	0.61054	0.50813
consistency	olfactory transduction(kegg)	0.00031	0.6714	0.53803
consistency	vibrio cholerae infection(kegg)	5.20E-06	0.93300	0.84252

Table 3. *A sample of the significant pathways from Wilcoxon rank sum test in TCGA tumor female v. TCGA tumor male comparison*. Activity and consistency values were calculated using PathOlogist, then a Wilcoxon rank sum test was performed comparing male and female samples in TCGA HCC tissue. A p-value threshold of <=0.05 was used to determine significance.

score type	pathway	p-value	male mean	female mean
activity	d4gdi signaling pathway(biocarta)	0.0248641	0.86252252	0.8394305785
activity	androgen and estrogen metabolism(kegg)	0.0449593	0.51102450	0.4634157025
activity	bile acid biosynthesis(kegg)	0.0001439	0.78777470	0.7613785124
activity	bile secretion(kegg)	0.0416733	0.78290553	0.792838843
activity	caffeine metabolism(kegg)	6.71E-06	0.72725652	0.6600272727
activity	chemical carcinogenesis(kegg)	0.0080530	0.54383201	0.4852785124
activity	histidine metabolism(kegg)	5.28E-05	0.73204940	0.7071347107
activity	pyruvate metabolism(kegg)	0.0081311	0.79177035	0.7750752066
activity	tgf-beta signaling pathway(kegg)	0.0071353	0.63219565	0.6162942149
activity	pentose phosphate pathway(reactome)	2.96E-05	0.67816917	0.6074123967
activity	abacavir transport and metabolism(reactome)	0.0001372	0.76148774	0.7235884298
activity	signaling by tgf-beta family members(reactome)	0.0109980	0.08419604	0.09484297521
consistency	mechanism of acetaminophen activity and toxicity(biocarta)	5.55E-06	0.58742687	0.5228876033
consistency	androgen and estrogen metabolism(kegg)	0.0449593	0.51102450	0.4634157025
consistency	ascorbate and aldarate metabolism(kegg)	9.54E-07	0.50617312	0.4240173554
consistency	bile acid biosynthesis(kegg)	0.0001439	0.78777470	0.7613785124
consistency	pyruvate metabolism(kegg)	0.0081311	0.79177035	0.7750752066
consistency	histidine metabolism(kegg)	5.28E-05	0.73204940	0.7071347107
consistency	pentose phosphate pathway(reactome)	2.96E-05	0.67816917	0.6074123967
consistency	abacavir transport and metabolism(reactome)	0.00013723 15179	0.76148774	0.7235884298

Figure 1. *Mean activity values for males and females are highly correlated with each other in HCC.* Activity values from TCGA samples in HCC were averaged across each sex then plotted for each of the 1290 pathways. A linear regression model was overlaid to show the degree of correlation.

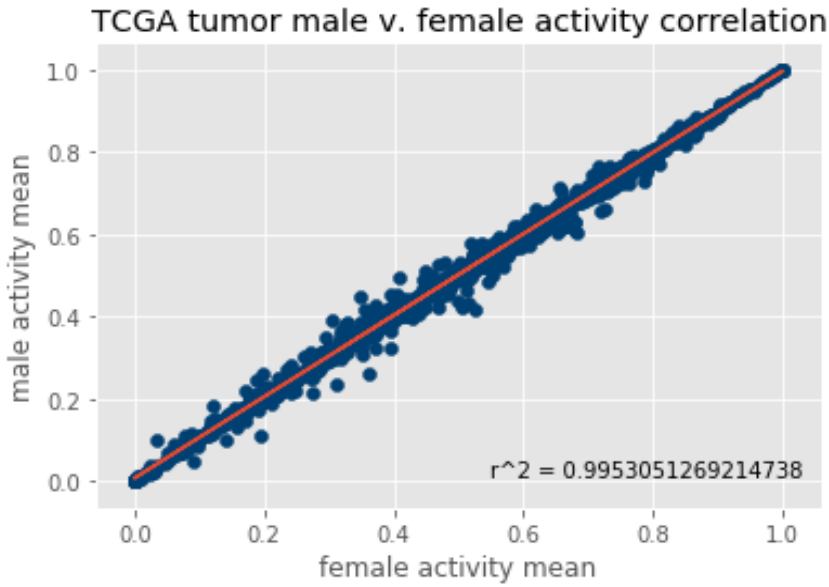


Figure 2. *Venn-diagram of the overlap of significantly different pathway activity scores between within tissue analyses.* The activity scores were analyzed using the Wilcoxon rank sum test comparing male and female samples. Each tissue type was calculated separately, and p-values were Bonferroni adjusted with a threshold of <=0.05.

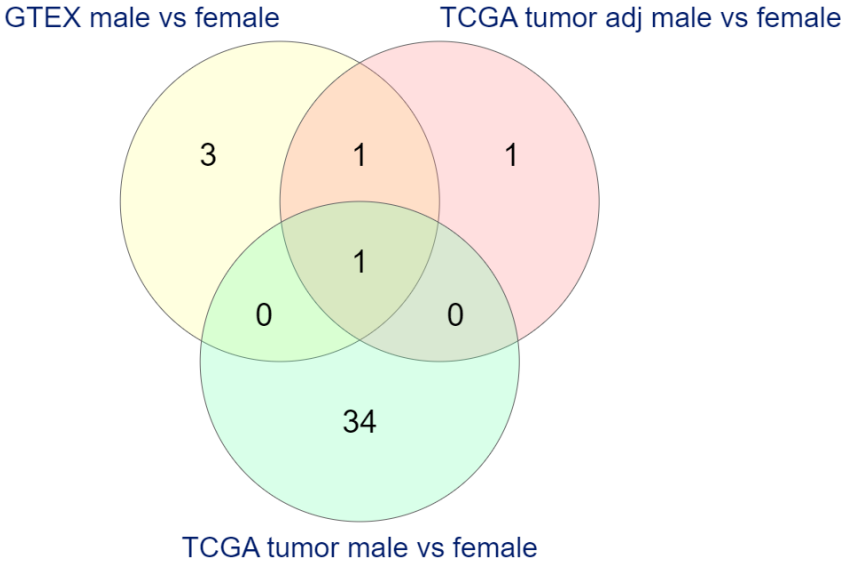


Figure 3. *Scatter plot of activity values for wnt signaling pathway (kegg) in GTEx normal liver tissue.* Activity scores were calculated using PathOlogist, comparing females and males in GTEx normal liver tissue. Females and males are colored separately with mean lines to show their central tendency.

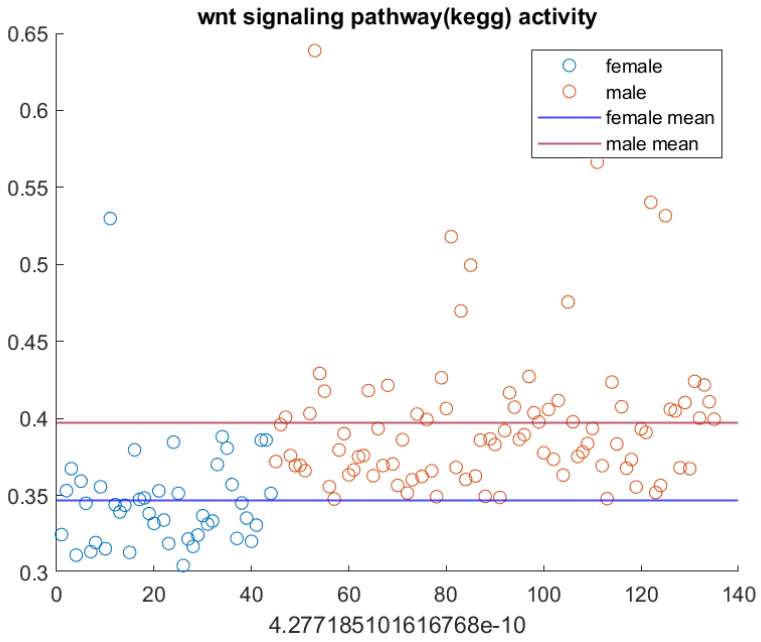


Figure 4. *Histogram of activity scores with overlaid mixed gaussian distribution for long term potentiation (kegg) for the GTEx female v. male comparison.* Activity scores were calculated using PathOlogist. A mixed gaussian distribution was calculated and overlaid along with each component to show active and inactive distributions.

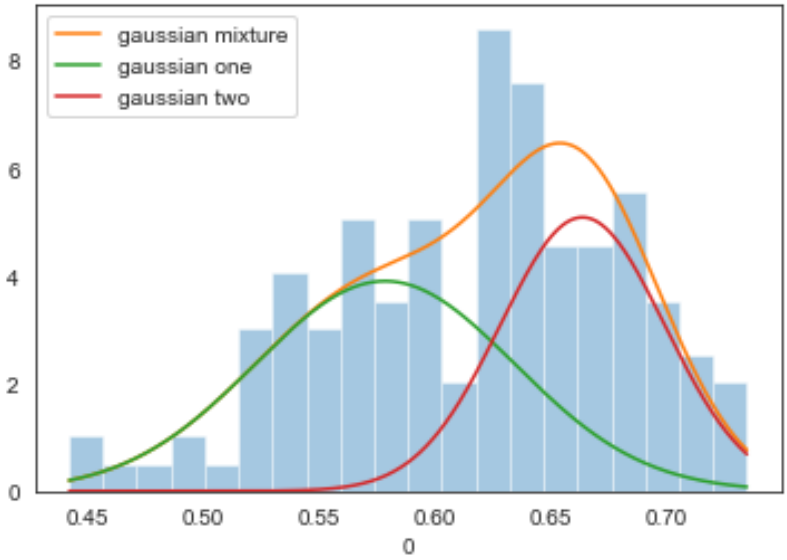


Figure 5. *Bar graph of the proportion of female and male active samples for the wnt signaling pathway (kegg).* Classification data from GTEx female v male was calculated then plotted for proportion of active samples in females and males.

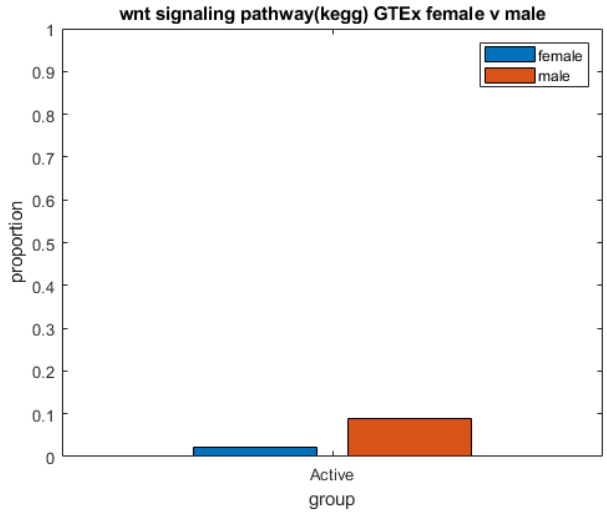
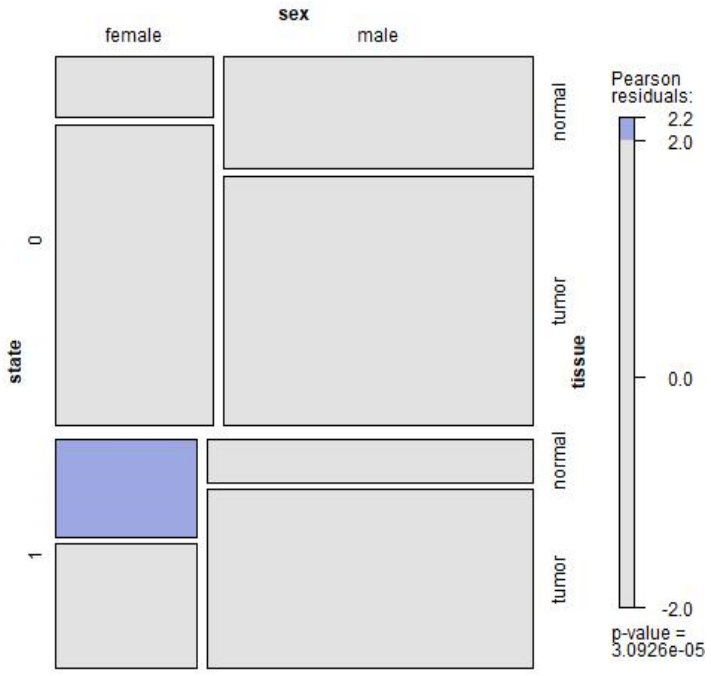


Figure 6. *Mosaic plot of the log-linear model for chromatin modifying enzymes (reactome).* Pathway consistency classification data comparing females and males while adjusting for normal liver tissue and HCC was analyzed in a joint-independence model. Females are overrepresented in the active distribution (‘1’) for normal liver tissue.



RESULTS

We identified sex differences in pathway activity and consistency in normal, tumor-adjacent, and HCC liver tissue to uniquely characterize the biological processes that drive the sex-biases observed in HCC occurrence and etiology.

1. Males show higher activity than females for multiple pathways in normal and tumor adjacent liver tissue

In normal liver tissue, we identified 5 pathways whose activity scores were different between males and females using the Wilcoxon rank sum test. These pathways were found to regulate the cell cycle, proliferation, and differentiation, notably the *wnt signaling pathway (kegg)* and *eukaryotic translation elongation (kegg)*. For tumor adjacent tissue we identified 3 pathways, 2 of which were also identified in normal liver tissue, *long-term potentiation (kegg)* and *vibrio cholerae infection* (*kegg)*. For all 6 unique pathways identified from both tissue types, males had a higher mean activity score than females (Table 1; Table 2).

To further analyze the distribution of the pathways, each sample was classified as 'active' or 'inactive' based on our classification algorithm. It was revealed that males had a higher proportion of active samples for all pathways identified from both tissue types.

2. Sex-biased patterns of pathway activity in HCC

The analysis of HCC revealed the most substantial quantity of pathways with sex differences in activity scores. We identified 34 pathways with sex-biased activity in HCC using the Wilcoxon rank sum test, notably *tgf-beta signaling pathway (kegg)* and

androgen and estrogen metabolism (kegg) (Table 3). Unlike normal and tumor adjacent tissue, there was no observed trend of male activity in relation to females in HCC. Furthermore, classification analysis of HCC samples revealed that higher mean activity scores correlated with a larger proportion of active samples for all significant pathways.

3. Sex differences observed in pathway consistency across all tissue types

Sex differences were observed in pathway consistency for normal tissue, tumor adjacent tissue, and HCC using the Wilcoxon rank sum test. HCC had the highest quantity of significant pathways with 46 identified (Table 3). 7 pathways were identified in normal liver tissue and 4 pathways were identified in tumor adjacent tissue (Figure 2).

Chi-squared analysis in HCC tumor tissue comparing classification data for females and males identified two more pathways with sex differences in consistency scores, *toll-like receptor pathway (biocarta)* and *dicer pathway (biocarta)*.

4. Cross-tissue analysis reveals sex differences in liver tissue

To further examine the sex differences driving HCC etiology, we tested for significant differences between normal, tumor adjacent, and tumor liver tissue using chi-squared analysis performed in sex-combined and sex specific comparisons (see Appendix II). In the normal v. tumor adjacent combined analysis of female and male samples, we detected 647 and 609 pathways in activity and consistency, respectively. In female and male specific analyses for activity, we detected 403 and 502 pathways, respectively. For consistency, we detected 343 and 439 significant pathways, respectively. In sex-specific analyses for activity and consistency, we detected 367 and 313 pathways that were shared

between sexes. Of the identified pathways, 7 were male-specific in activity. In consistency, 2 pathways were female-specific and 5 were male-specific.

In normal v. tumor combined analysis of female and male samples, we detected 699 and 755 pathways in activity and consistency, respectively. For activity in female and male specific analyses, we detected 489 and 663 pathways, respectively. For consistency in female and male specific analyses, we detected 462 and 675 pathways, respectively. Of the identified pathways in activity, 3 were female-specific and 20 were male-specific. In consistency, 4 pathways were male-specific.

In tumor adjacent v. tumor combined analysis of female and male samples, we detected 687 and 640 pathways in activity and consistency, respectively. For activity in female and male specific analyses, we detected 374 and 490 pathways, respectively. For consistency in female and male specific analyses, we detected 345 and 440, respectively. Of the identified pathways in activity, 10 were female-specific and 5 were male-specific. In consistency, 8 were female-specific and 7 were male-specific.

5. Cross-tissue log-linear models reveal sex differences in pathway consistency

Pathways that were significant between sex (eg. female and male) while adjusting for tissue type (eg. normal and tumor) were identified using log-linear modeling of the sex-combined classification data. 2 pathways were identified using this method comparing females and males in normal liver tissue and HCC for pathway consistency, *chromatin modifying enzymes (reactome)* and *eukaryotic translation initiation (reactome)*. In *chromatin modifying enzymes (reactome)*, females were overrepresented

in the active distribution for normal liver tissue with Pearson residuals > 2.0 (Figure 6). Females in *eukaryotic translation initiation (reactome)* were underrepresented in the active distribution for normal liver tissue (Pearson residuals < 2.0) and overrepresented in the inactive distribution for HCC (Pearson residuals > 2.0).

DISCUSSION

We identified pathways that were different in normal, tumor adjacent, and tumor tissue types, indicating that male and female HCC are partially driven by different mechanisms and processes. While sex-biased differences in pathway activity or consistency may contribute to the sex-differences in cancer-occurrence, they are suggestive of distinct changes in biological mechanisms and processes between female and male HCC. Furthermore, a pathway that is significantly different between sexes in every tissue type may not be as biologically significant in the context of HCC etiology as a pathway that is significant in a single tissue type due to inherent biological differences between females and males.

Pathways that are identified exclusively in normal liver tissue have a variety of implications for sex differences in liver tissue. Factors such as sex chromosomes or hormonal regulation can affect the biological mechanisms between sexes, so it is important to reveal the origin of any differences in pathway activity or consistency.

Tumor adjacent tissue is representative of normal liver tissue; however, it is taken from an individual with HCC and is proximal to the tumor. As such, its expression may reflect constitutional genetic and environmental factors that are critical to the

development of cancer. Moreover, factors produced by the tumor itself (angiogenesis factors, immune modifying factors, etc) may alter the expression in this tissue.

Pathways that are uniquely different in HCC should provide insight into the differences between male and female tumor biology. A pathway's exclusivity to HCC makes it difficult to determine its role in HCC etiology; however, it can give researchers a framework for the development of personalized treatments. Additionally, sex-based analysis between normal and tumor tissue can provide insights into the mutational differences driving tumorigenesis.

From the results presented, we can identify pathways that reflect sex differences in healthy liver tissue, tumor-adjacent tissue, and HCC. The differences found in HCC were the largest compared to the other tissue types in both activity and consistency. These sex differences point to biological mechanisms within the liver which may translate to the sex-biases observed in HCC occurrence and mortality.

1. Male activity of translation factors and signaling pathways may drive sex-biased carcinogenesis in normal and tumor adjacent liver tissue

In normal and tumor adjacent liver tissue, we observed higher activity in male translation factors. Translation factors have garnered a lot of attention for their role in the onset and progression of various cancers (Hao et al. 2020). Eukaryotic mRNA translation is a complex process that includes four phases (initiation, elongation, termination, and ribosome recycling). Dysregulated mRNA translation is a common feature of carcinogenesis. Various oncogenic and tumor suppressive genes interact with translation

machinery, making the components of the translation promising therapeutic targets. Males showed a higher activity than females in *eukaryotic translation elongation (reactome)*. Additionally, our results show sex differences in consistency for *eukaryotic translation initiation (reactome)* in cross-tissue analysis between normal and tumor tissue. These sex differences observed in pathways related to translation factors could prove to be promising targets for therapeutic interventions.

We also observed high activity in male signaling pathways in normal liver tissue. The *wnt signaling pathway (kegg)* is implicated in many physiological processes, including development, immune response, tissue homeostasis, and tissue regeneration. This pathway is a key factor in the development of hepatocytes and the liver's ability to regenerate. The *wnt signaling pathway (kegg)* outlines the process by which beta-catenin is translocated into the nucleus (Wang et al. 2019). This translocation promotes the proliferation of hepatocytes, which can be a contributing factor to HCC. Additionally, it promotes the binding of t-cell transcription factors which support sex differences in the immune system. Activation of Wnt signaling pathways have been observed in multiple cancer types, making it an ideal target for therapeutics (Jung and Park 2020). Interestingly, we observed that males have a higher activity and consistency than females, but only in normal liver tissue. In the context of HCC etiology, increased activity of the pathway may be a driving factor of male-biased incidence.

2. Sex differences in metabolic pathway behaviors may underlie sex-biased occurrence and etiology

We identified metabolic pathways in HCC with sex-biased activity. HCC onset and progression is frequently accompanied by alterations of metabolic pathways, leading to dysregulation of metabolism. This is because the liver is a major regulator in the clearance of toxins and balancing of glucose, lipid, and amino acid uptake. It also manages metabolism throughout the whole today to maintain homeostasis. Some of these metabolic pathways are of particular interest in HCC.

Androgen and estrogen metabolism (kegg) is a key factor in regulating liver homeostasis and function (Shen and Haifei 2015). The liver expresses both androgen and estrogen receptors and experimentally both androgens and estrogens have been implicated in stimulating hepatocyte proliferation and may act as liver tumor inducers or promoters (GIANNITRAPANI et al. 2006). The male-biased activity of this pathway may be a factor in HCC etiology. As a therapeutic target, certain studies have shown that neither androgen nor estrogen inhibitors are associated with a recession of cancer development, indicating that steroid biosynthesis may be a better target for cancer treatment. *Chemical carcinogenesis (kegg)* is another pathway that we identified to have male-biased activation. This pathway contains many genes associated with steroid biosynthesis, possibly revealing candidates for a valid target in cancer treatment.

The mechanism of acetaminophen activity and toxicity (biocarta) is commonly associated with liver failure (Ramachandran and Jaeschke 2017). The overdose of acetaminophen is the most common cause of liver failure due to its narrow therapeutic

window and prescription of high doses. Excess damage to the liver may eventually lead to cirrhosis which increases the risk of developing HCC. Differences in the activity of this pathway provide insight to biological susceptibility or resistance in the toxicity of acetaminophen. Relevant literature suggests that the female preponderance of drug-induced liver injury may be related to sex-based differences in the expression of genes that affect liver metabolic function and pathophysiology (Waxman and Holloway 2009). While this pathway may not be indicative of the sex-biased occurrence of liver in males, further research may reveal important biological functions affected by this pathway.

Bile acid biosynthesis (kegg) and *bile secretion (kegg)* were found to have differences between sexes. Males had a higher activity in *bile acid biosynthesis (kegg),* but a lower activity in *bile secretion (kegg)* in relation to females. This may indicate that males have higher levels of bile acid in the liver due to both higher production and lower secretion, which is supported by animal models (Bennion et al. 1978). Bile acid is a water-soluble steroid synthesized in the hepatocytes of the liver. It is critical for digestion and absorption of fats and fat-soluble vitamins in the small intestine; however, in high levels it can be very dangerous (Wang et al. 2013). Bile acid has been found to be a potential carcinogen and deregulation of bile acid homeostasis has been linked to HCC formation (Cameron et al. 1982; Gupta et al. 2004; Jean-Louis et al. 2006) . The potential of increased levels of bile acid in male HCC has implications for its etiology.

Many pathways associated with carbohydrate and amino-acid metabolism such as *histidine metabolism (kegg), pyruvate metabolism (kegg),* and *pentose phosphate pathway (reactome)* were found to be significantly different between sexes. Relevant

literature shows that energy metabolism has great potential as a target for anticancer drugs (Rodríguez-Enríquez et al. 2014; Tao et al. 2015). The metabolic profile in healthy tissue is vastly different than in cancerous tissue and there is further variation between organ systems (Howarth et al. 2008; Nilsson et al. 2020). The results presented here shows that sex may be another important source of variation in metabolic profiles in addition to tissue types.

3. Differences in activation of signaling and cell-cycle pathways in HCC may drive sex-biased tumorigenesis

D4-GDI signaling pathway (biocarta) has an important role in apoptotic cells. Changes in apoptosis is a notable factor in the development of many cancer types, including HCC as well as a regulator of many immune cell types. D4-GDI (a member of Rho family of GDP Dissociation Inhibitors, or RhoGDIs) is a negative regulator of the Ras related Rho Family of GTPases. Rho GTPases promote cytoskeletal and membrane changes associated with apoptotic cell death, so the removal of the D4-GDI block through its cleavage is important for inducing apoptosis (Essmann et al. 2000). RhoGDIs have also been a target of interest in certain cancer-type therapies including breast, bladder, and pancreatic cancer for their role in carcinogenesis and tumor progression (Harding and Theodorescu 2010). The pattern of signaling in a RhoGDI pathway is highly variable between tissues, so therapeutic intervention will be highly dependent on the cancer type or even sub-type. The results presented here have implications for targeted treatment of male and female HCC.

TGF-beta signaling pathway (kegg) is involved in every stage of liver disease progression, including HCC (Fabregat et al. 2016). During early stages of tumorigenesis, it acts as a tumor suppressor, while in later stages it acts with a pro-tumorigenic role, promoting invasiveness and metastasis once cells become resistant to its suppressor effects (Fabregat and Caballero-Díaz 2018). TGF-beta is one of the strongest inducers of epithelial-mesenchymal transition (EMT) which promotes tumor heterogeneity and apoptotic resistance. Pathway analysis of HCC samples show that males have a higher mean activity of TGF-beta signaling pathway than females. A higher activity within this pathway could have implications for different rates of tumor progression and invasiveness in males.

4. Cross tissue analysis reveals sex-specific differences in immune pathways between normal and HCC liver tissue

Notable findings for cross-tissue analysis was the identification of immune pathways in sex-specific analysis. Immune pathways such as *il-12 signaling mediated by stat4 (nci/nature), il-7 signal transduction (biocarta),* and *p75nrt signals via nf-kb (reactome)* had different activity between tissues for males in cross tissue analysis between normal, tumor adjacent, and HCC liver tissue, but not females. Characterization of immune profiles is important to the development of targeted immunotherapies, so sex differences in pathway activity implies a need for sex-specific approaches in the development of immunotherapy in HCC. The results we present lay a foundation for future research into considering sex as a variable for clinical research in the context of targeted immunotherapy.

CONCLUSION

Here we identified biological pathways with sex differences in HCC. Notably, we identified pathways related to metabolism, sex-hormones, bile acid, signaling, and translation factors. These pathways were shown in previous literature to be important to the risk and severity of HCC. Importantly, their detection shows the power that pathway analysis has in characterizing cancer subtypes. Our research provides a framework for future studies to create therapeutic interventions or screening tools for HCC or other sex-biased cancers. Future research would be needed to better understand the effects of these functions and their importance as a biomarker or target for cancer treatment

REFERENCES

Bennion, L. J., E. Drobny, W. C. Knowler, R. L. Ginsberg, M. B. Garnick, R. D. Adler, and W. C. Duane. 1978. “Sex Differences in the Size of Bile Acid Pools.” Metabolism: Clinical and Experimental 27 (8): 961–69. https://doi.org/10.1016/0026-0495(78)90140-3.

Bolger, Anthony M., Marc Lohse, and Bjoern Usadel. 2014. “Trimmomatic: A Flexible Trimmer for Illumina Sequence Data.” Bioinformatics 30 (15): 2114–20. https://doi.org/10.1093/bioinformatics/btu170.

Cameron, Ross G., 2. Katsumi Imaida, Hiroyuki Tsuda, and Nobuyuki Ito. 1982. “Promotive Effects of Steroids and Bile Acids on Hepatocarcinogenesis Initiated by Diethylnitrosamine1.” Cancer Research 42: 2426–28. http://cancerres.aacrjournals.org/content/42/6/2426.

Charni-Natan, Meital, Ronit Aloni-Grinstein, Etty Osher, and Varda Rotter. 2019. “Liver and Steroid Hormones-Can a Touch of p53 Make a Difference?” Frontiers in Endocrinology 10 (June): 374. https://doi.org/10.3389/fendo.2019.00374.

Clocchiatti, Andrea, Elisa Cora, Yosra Zhang, and G. Paolo Dotto. 2016. “Sexual Dimorphism in Cancer.” Nature Reviews. Cancer 16 (5): 330–39. https://doi.org/10.1038/nrc.2016.30.

Essmann, F., T. Wieder, A. Otto, E. C. Müller, B. Dörken, and P. T. Daniel. 2000. “GDP Dissociation Inhibitor D4-GDI (Rho-GDI 2), but Not the Homologous Rho-GDI 1, Is Cleaved by Caspase-3 during Drug-Induced Apoptosis.” Biochemical Journal 346 Pt 3 (March): 777–83. https://doi.org/10.1042/0264-6021:3460777.

Fabregat, Antonio, Konstantinos Sidiropoulos, Guilherme Viteri, Oscar Forner, Pablo Marin-Garcia, Vicente Arnau, Peter D’Eustachio, Lincoln Stein, and Henning Hermjakob. 2017. “Reactome Pathway Analysis: A High-Performance in-Memory Approach.” BMC Bioinformatics 18 (1): 142. https://doi.org/10.1186/s12859-017-1559-2.

Fabregat, Isabel, and Daniel Caballero-Díaz. 2018. “Transforming Growth Factor-β-Induced Cell Plasticity in Liver Fibrosis and Hepatocarcinogenesis.” Frontiers in Oncology 8 (September): 357. https://doi.org/10.3389/fonc.2018.00357.

Fabregat, Isabel, Joaquim Moreno-Càceres, Aránzazu Sánchez, Steven Dooley, Bedair Dewidar, Gianluigi Giannelli, Peter Ten Dijke, and IT-LIVER Consortium. 2016. “TGF-β Signalling and Liver Disease.” The FEBS Journal 283 (12): 2219–32. https://doi.org/10.1111/febs.13665.

Ghaffari, Pouyan, Adil Mardinoglu, Anna Asplund, Saeed Shoaie, Caroline Kampf, Mathias Uhlen, and Jens Nielsen. 2015. “Identifying Anti-Growth Factors for Human Cancer Cell Lines through Genome-Scale Metabolic Modeling.” Scientific Reports 5 (1): 8183. https://doi.org/10.1038/srep08183.

Global Burden of Disease Cancer Collaboration, Christina Fitzmaurice, Christine Allen, Ryan M. Barber, Lars Barregard, Zulfiqar A. Bhutta, Hermann Brenner, et al. 2017. “Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 32 Cancer Groups, 1990 to 2015: A Systematic Analysis for the Global Burden of Disease Study.” JAMA Oncology 3 (4): 524–48. https://doi.org/10.1001/jamaoncol.2016.5688.

Greenblum, Sharon I., Sol Efroni, Carl F. Schaefer, and Ken H. Buetow. 2011. “The PathOlogist: An Automated Tool for Pathway-Centric Analysis.” BMC Bioinformatics 12 (May): 133. https://doi.org/10.1186/1471-2105-12-133.

Gupta, Seema, Ramesh Natarajan, Shawn G. Payne, Elaine J. Studer, Sarah Spiegel, Paul Dent, and Phillip B. Hylemon. 2004. “Deoxycholic Acid Activates the c-Jun N-Terminal Kinase Pathway via FAS Receptor Activation in Primary Hepatocytes: ROLE OF ACIDIC SPHINGOMYELINASE-MEDIATED CERAMIDE GENERATION IN FAS RECEPTOR ACTIVATION *.” The Journal of Biological Chemistry 279 (7): 5821–28. https://doi.org/10.1074/jbc.M310979200.

Guy, Jennifer, and Marion G. Peters. 2013. “Liver Disease in Women: The Influence of Gender on Epidemiology, Natural History, and Patient Outcomes.” Gastroenterology & Hepatology 9 (10): 633–39. https://www.ncbi.nlm.nih.gov/pubmed/24764777.

Hao, Peiqi, Jiaojiao Yu, Richard Ward, Yin Liu, Qiao Hao, Su An, and Tianrui Xu. 2020. “Eukaryotic Translation Initiation Factors as Promising Targets in Cancer Therapy.” Cell Communication and Signaling: CCS 18 (1): 175. https://doi.org/10.1186/s12964-020-00607-9.

Harding, Michael A., and Dan Theodorescu. 2010. “RhoGDI Signaling Provides Targets for Cancer Therapy.” European Journal of Cancer 46 (7): 1252–59. https://doi.org/10.1016/j.ejca.2010.02.025.

Heinlein, Cynthia A., and Chawnshang Chang. 2004. “Androgen Receptor in Prostate Cancer.” Endocrine Reviews 25 (2): 276–308. https://doi.org/10.1210/er.2002-0032.

Howarth, Nancy C., Suzanne P. Murphy, Lynne R. Wilkens, Brian E. Henderson, and Laurence N. Kolonel. 2008. “The Association of Glycemic Load and Carbohydrate Intake with Colorectal Cancer Risk in the Multiethnic Cohort Study.” The American Journal of Clinical Nutrition 88 (4): 1074–82. https://doi.org/10.1093/ajcn/88.4.1074.

Iyer, Janaki K., Mamta Kalra, Anil Kaul, Mark E. Payton, and Rashmi Kaul. 2017. "Estrogen Receptor Expression in Chronic Hepatitis C and Hepatocellular Carcinoma Pathogenesis." World Journal of Gastroenterology: WJG 23 (37): 6802–16. https://doi.org/10.3748/wjg.v23.i37.6802.

Jean-Louis, Samira, Sandeep Akare, M. Ahad Ali, Eugene A. Mash, Emmanuelle Meuillet, and Jesse D. Martinez. 2006. "Deoxycholic Acid Induces Intracellular Signaling through Membrane Perturbations *." The Journal of Biological Chemistry 281 (21): 14948–60. https://doi.org/10.1074/jbc.M506710200.

Jung, Youn-Sang, and Jae-Il Park. 2020. "Wnt Signaling in Cancer: Therapeutic Targeting of Wnt Signaling beyond β-Catenin and the Destruction Complex." Experimental & Molecular Medicine 52 (2): 183–91. https://doi.org/10.1038/s12276-020-0380-6.

Kanehisa, Minoru, Miho Furumichi, Mao Tanabe, Yoko Sato, and Kanae Morishima. 2017. "KEGG: New Perspectives on Genomes, Pathways, Diseases and Drugs." Nucleic Acids Research 45 (D1): D353–61. https://doi.org/10.1093/nar/gkw1092.

Kapp, Lee D., and Jon R. Lorsch. 2004. "The Molecular Mechanics of Eukaryotic Translation." Annual Review of Biochemistry 73: 657–704. https://doi.org/10.1146/annurev.biochem.73.030403.080419.

Konduri, Santhi, Maharaj Singh, George Bobustuc, Richard Rovin, and Amin Kassam. 2020. "Epidemiology of Male Breast Cancer." Breast 54 (December): 8–14. https://doi.org/10.1016/j.breast.2020.08.010.

Law, Charity W., Yunshun Chen, Wei Shi, and Gordon K. Smyth. 2014. "Voom: Precision Weights Unlock Linear Model Analysis Tools for RNA-Seq Read Counts." Genome Biology 15 (2): R29. https://doi.org/10.1186/gb-2014-15-2-r29.

Li, Constance H., Syed Haider, Yu-Jia Shiah, Kevin Thai, and Paul C. Boutros. 2018. "Sex Differences in Cancer Driver Genes and Biomarkers." Cancer Research 78 (19): 5527–37. https://doi.org/10.1158/0008-5472.CAN-18-0362.

Nilsson, Avlant, Jurgen R. Haanstra, Martin Engqvist, Albert Gerding, Barbara M. Bakker, Ursula Klingmüller, Bas Teusink, and Jens Nielsen. 2020. "Quantitative Analysis of Amino Acid Metabolism in Liver Cancer Links Glutamate Excretion to Nucleotide Synthesis." Proceedings of the National Academy of Sciences of the United States of America 117 (19): 10294–304. https://doi.org/10.1073/pnas.1919250117.

Nishimura, Darryl. 2001. "BioCarta." Biotech Software & Internet Report 2 (3): 117–20. https://doi.org/10.1089/152791601750294344.

OuYang, P-Y, L-N Zhang, X-W Lan, C. Xie, W-W Zhang, Q-X Wang, Z. Su, J. Tang, and F-Y Xie. 2015. “The Significant Survival Advantage of Female Sex in Nasopharyngeal Carcinoma: A Propensity-Matched Analysis.” British Journal of Cancer 112 (9): 1554–61. https://doi.org/10.1038/bjc.2015.70.

Patro, Rob, Geet Duggal, Michael I. Love, Rafael A. Irizarry, and Carl Kingsford. 2017. “Salmon Provides Fast and Bias-Aware Quantification of Transcript Expression.” Nature Methods 14 (4): 417–19. https://doi.org/10.1038/nmeth.4197.

Petrick, Jessica L., Megan Braunlin, Mathieu Laversanne, Patricia C. Valery, Freddie Bray, and Katherine A. McGlynn. 2016. “International Trends in Liver Cancer Incidence, Overall and by Histologic Subtype, 1978-2007.” International Journal of Cancer. Journal International Du Cancer 139 (7): 1534–45. https://doi.org/10.1002/ijc.30211.

Ramachandran, Anup, and Hartmut Jaeschke. 2017. “Mechanisms of Acetaminophen Hepatotoxicity and Their Translation to the Human Pathophysiology.” Translational Research: The Journal of Laboratory and Clinical Medicine 3 (Suppl 1): 157–69. https://doi.org/10.18053/jctres.03.2017S1.002.

Rodríguez-Enríquez, Sara, Juan Carlos Gallardo-Pérez, Ileana Hernández-Reséndiz, Alvaro Marín-Hernández, Silvia C. Pacheco-Velázquez, Sayra Y. López-Ramírez, Franklin D. Rumjanek, and Rafael Moreno-Sánchez. 2014. “Canonical and New Generation Anticancer Drugs Also Target Energy Metabolism.” Archives of Toxicology 88 (7): 1327–50. https://doi.org/10.1007/s00204-014-1246-2.

Schaefer, Carl F., Kira Anthony, Shiva Krupa, Jeffrey Buchoff, Matthew Day, Timo Hannay, and Kenneth H. Buetow. 2009. “PID: The Pathway Interaction Database.” Nucleic Acids Research 37 (Database issue): D674–79. https://doi.org/10.1093/nar/gkn653.

Shen, Minqian, and Haifei Shi. 2015. “Sex Hormones and Their Receptors Regulate Liver Energy Homeostasis.” International Journal of Endocrinology 2015 (September): 294278. https://doi.org/10.1155/2015/294278.

Siegel, Rebecca L., Kimberly D. Miller, and Ahmedin Jemal. 2020. “Cancer Statistics, 2020.” CA: A Cancer Journal for Clinicians 70 (1): 7–30. https://doi.org/10.3322/caac.21590.

Tao, Cui, Jingchun Sun, W. Jim Zheng, Junjie Chen, and Hua Xu. 2015. “Colorectal Cancer Drug Target Prediction Using Ontology-Based Inference and Network Analysis.” Database: The Journal of Biological Databases and Curation 2015 (March). https://doi.org/10.1093/database/bav015.

Wands, Jack. 2007. “Hepatocellular Carcinoma and Sex.” The New England Journal of Medicine 357 (19): 1974–76. https://doi.org/10.1056/NEJMcibr075652.

Wang, Wenhui, Ron Smits, Haiping Hao, and Chaoyong He. 2019. “Wnt/β-Catenin Signaling in Liver Cancers.” Cancers 11 (7). https://doi.org/10.3390/cancers11070926.

Wang, Xichun, Xianghui Fu, Carl Van Ness, Zhipeng Meng, Xiaoxiao Ma, and Wendong Huang. 2013. “Bile Acid Receptors and Liver Cancer.” Current Pathobiology Reports 1 (1): 29–35. https://doi.org/10.1007/s40139-012-0003-6.

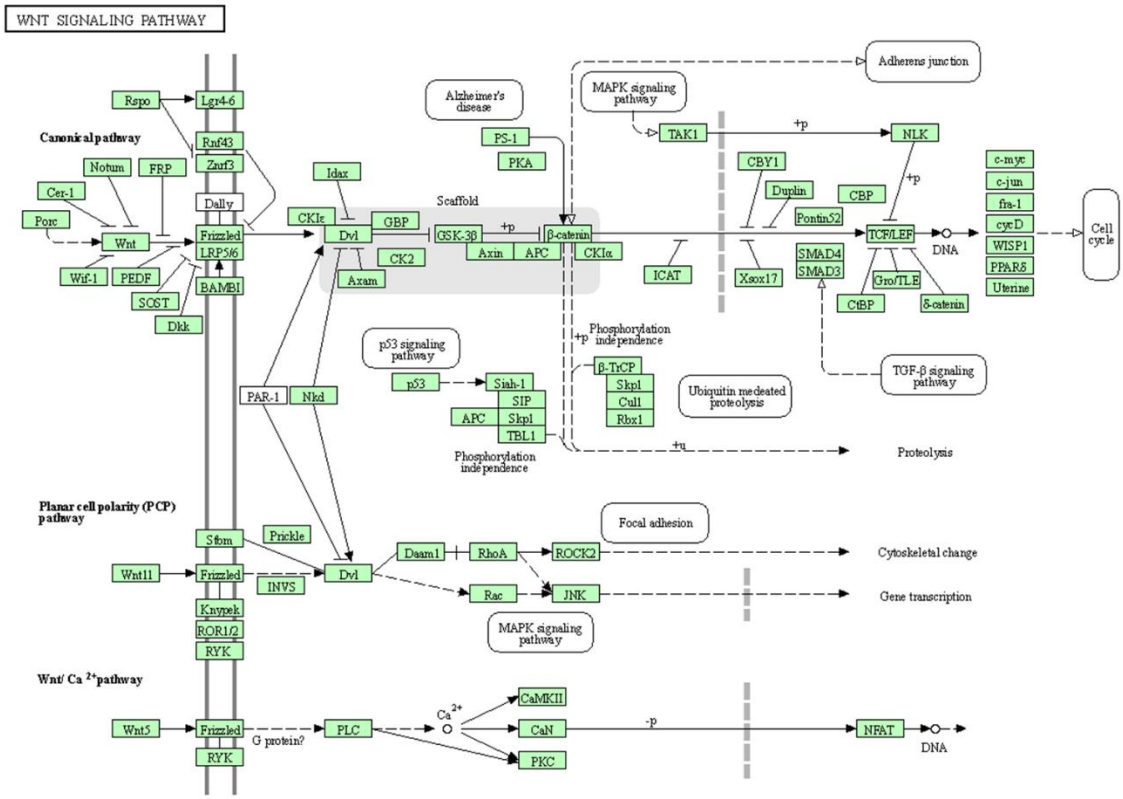
Waxman, David J., and Minita G. Holloway. 2009. “Sex Differences in the Expression of Hepatic Drug Metabolizing Enzymes.” Molecular Pharmacology 76 (2): 215–28. https://doi.org/10.1124/mol.109.056705.

Wisnivesky, Juan P., and Ethan A. Halm. 2007. “Sex Differences in Lung Cancer Survival: Do Tumors Behave Differently in Elderly Women?” Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 25 (13): 1705–12. https://doi.org/10.1200/JCO.2006.08.1455.

Yager, James D., and Nancy E. Davidson. 2006. “Estrogen Carcinogenesis in Breast Cancer.” The New England Journal of Medicine 354 (3): 270–82. https://doi.org/10.1056/NEJMra050776.

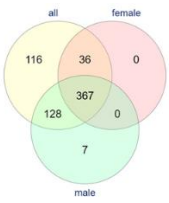
APPENDIX I

WNT SIGNALING PATHWAY (KEGG) INTERACTION DIAGRAM

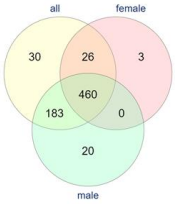

WNT SIGNALING PATHWAY
Canonical pathway
Rspo
Lgr4-6
Rnf43
Znrf3
Notum
FRP
Cer-1
Porc
Wnt
Frizzled
LRP5/6
Wif-1
PEDF
SCGT
Dkk
Dally
BAMBI
Idax
CKIε
Dvl
GBP
CK2
Axam
Scaffold
GSK-3β
+p
β-catenin
Axin
APC
CKIα
Alzheimer's disease
PS-1
PKA
MAPK signaling pathway
TAK1
Adherens junction
NLK
CBY1
Duplin
CBP
Pontin52
TCF/LEF
DNA
c-myc
c-jun
fra-1
cycD
WISP1
PPAR δ
Uterine
Cell cycle
ICAT
Xsox17
SMAD4
SMAD3
Gro/TLE
CtBP
δ-catenin
TGF-β signaling pathway
p53 signaling pathway
p53
Siah-1
SIP
Skp1
TBL1
Phosphorylation independence
β-TrCP
Cul1
Rbx1
Ubiquitin mediated proteolysis
+u
Proteolysis
PAR-1
Nkd
Planar cell polarity (PCP) pathway
Sfrm
Prickle
Wnt11
Frizzled
INVS
Knypek
ROR1/2
RYK
Daam1
RhoA
ROCK2
Focal adhesion
Cytoskeletal change
Rac
JNK
Gene transcription
MAPK signaling pathway
Wnt/ Ca 2+pathway
Wnt5
Frizzled
RYK
G protein?
PLC
Ca 2+
CaMKII
CaN
PKC
-p
NFAT
DNA

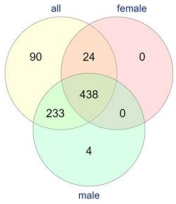
APPENDIX II

CROSS TISSUE CHI-SQUARED ANALYSIS: VENN DIAGRAMS AND SEX-SPECIFIC TABLES

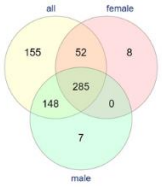


GTEx v TCGA tumor adjacent





GTEx v TCGA tumor



TCGA tumor adjacent v TCGA tumor

sex	**normal v tumor**	pathway
male	activity	cdc25 and chk1 regulatory pathway in response to dna damage(biocarta)
male	activity	hedgehog signaling events mediated by gli proteins(nci/nature)
male	activity	il12 signaling mediated by stat4(nci/nature)
male	activity	il-7 signal transduction(biocarta)
male	activity	regulation of cytoplasmic and nuclear smad2/3 signaling(nci/nature)
male	activity	vegfr3 signaling in lymphatic endothelium(nci/nature)
male	activity	acute myeloid leukemia(kegg)
male	activity	cholinergic synapse(kegg)
male	activity	**d-glutamine and d-glutamate metabolism(kegg)**
male	activity	legionellosis(kegg)
male	activity	**linoleic acid metabolism(kegg)**
male	activity	g2/m checkpoints(reactome)
male	activity	dna damage bypass(reactome)
male	activity	hyaluronan metabolism(reactome)
male	activity	fgfr1 ligand binding and activation(reactome)
male	activity	gpcr ligand binding(reactome)
male	activity	biosynthesis of dpa-derived spms(reactome)
male	activity	regulation of pten mrna translation(reactome)
male	activity	death receptor signalling(reactome)
male	activity	nucleosome assembly(reactome)
male	consistency	**d-glutamine and d-glutamate metabolism(kegg)**
male	consistency	**linoleic acid metabolism(kegg)**
male	consistency	response to metal ions(reactome)
male	consistency	eukaryotic translation elongation(reactome)
female	activity	cytokine-cytokine receptor interaction(kegg)
female	activity	gnrh signaling pathway(kegg)
female	activity	olfactory transduction(kegg)

sex	**tumor adjacent v tumor**	pathway
male	activity	**alanine and aspartate metabolism(kegg)**
male	activity	**galactose metabolism(kegg)**
male	activity	glycosaminoglycan degradation(kegg)
male	activity	**systemic lupus erythematosus(kegg)**
male	activity	**vibrio cholerae infection(kegg)**
male	consistency	atm pathway(nci/nature)
male	consistency	**alanine and aspartate metabolism(kegg)**
male	consistency	axon guidance(kegg)
male	consistency	**galactose metabolism(kegg)**
male	consistency	**systemic lupus erythematosus(kegg)**
male	consistency	**vibrio cholerae infection(kegg)**
male	consistency	protein-protein interactions at synapses(reactome)
female	activity	**mechanism of acetaminophen activity and toxicity(biocarta)**
female	activity	**chemical carcinogenesis(kegg)**
female	activity	**glycine**
female	activity	**serine and threonine metabolism(kegg)**
female	activity	**lysine degradation(kegg)**
female	activity	phototransduction(kegg)
female	activity	**tryptophan metabolism(kegg)**
female	activity	metabolism of water-soluble vitamins and cofactors(reactome)
female	activity	**biosynthesis of epa-derived spms(reactome)**
female	activity	**biosynthesis of maresins(reactome)**
female	consistency	**mechanism of acetaminophen activity and toxicity(biocarta)**
female	consistency	**chemical carcinogenesis(kegg)**
female	consistency	**glycine**
female	consistency	**serine and threonine metabolism(kegg)**
female	consistency	**lysine degradation(kegg)**
female	consistency	**tryptophan metabolism(kegg)**
female	consistency	**biosynthesis of epa-derived spms(reactome)**
female	consistency	**biosynthesis of maresins(reactome)**

sex	**normal v tumor adjacent**	pathway
male	activity	overview of telomerase rna component gene hterc transcriptional regulation(biocarta)
male	activity	**bile acid biosynthesis(kegg)**
male	activity	complement and coagulation cascades(kegg)
male	activity	p75ntr signals via nf-kb(reactome)
male	activity	peptide ligand-binding receptors(reactome)
male	activity	g alpha (s) signalling events(reactome)
male	activity	**biosynthesis of maresins(reactome)**
male	consistency	**bile acid biosynthesis(kegg)**
male	consistency	gabaergic synapse(kegg)
male	consistency	vibrio cholerae infection(kegg)
male	consistency	antigen processing-cross presentation(reactome)
male	consistency	**biosynthesis of maresins(reactome)**
female	consistency	interleukin-1 signaling(reactome)
female	consistency	g alpha (q) signalling events(reactome)