Identification of key regulatory pathways and regulators in the pathogenesis of hepatitis C virus-induced hepatocellular carcinoma

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Abstract

Hepatitis virus infection is a leading cause of chronic liver diseases, including cirrhosis and hepatocellular carcinoma (HCC). However, the molecular mechanism by which hepatitis causes liver cancer remains unclear. Additionally, new biomarkers for diagnosis, prognosis and therapeutics are needed. Regulatory pathways play important roles in many pathogenic processes, and identifying the pathways by which hepatitis C virus (HCV) induces HCC may lead to better diagnosis and treatment. We employed a systematic approach to identify important regulatory pathways in this disease process, and found several important regulators. First, three networks were constructed based on the gene expression in patients with hepatitis alone, HCC alone, and hepatitis with HCC. A priority algorithm was used to extract the regulatory pathways from the networks, which were then scored based on the disease-related genetic information to identify key pathways. After integrating the regulatory pathways involved in the three networks, we found key regulatory genes, including EZH2 and hsa-miR-155-5p. Based on network analysis, it appeared that in HCC patients the abnormal expression of genes and miRNAs were mostly caused by abnormal expression of these key regulatory factors. This method may help researchers discover the potential pathogenic factors of HCC and could also yield new biomarkers for disease diagnosis.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths worldwide [1]. The disease is often diagnosed at an advanced stage and progresses rapidly. Therefore, early diagnosis is very important to improve the prognosis of patients. Currently, early clinical screening methods involve serum alpha fetoprotein (AFP) detection and liver ultrasound examination[2]. However, the sensitivity and specificity of markers such as AFP are marginal [3]. Additionally, ultrasound examination relies heavily on the subjective judgment of the operator, and conventional ultrasound often does not give results which can be used to conclusively identify liver lesions. Therefore, there is an urgent need to find more effective and accurate methods for screening for liver cancer. As the understanding of tumor biology improves, liquid biopsy will become an increasingly useful tool for early diagnosis. Risk factors for primary liver cancer include hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, aflatoxin B intake, alcohol consumption, cirrhosis and others. Among these risk factors, HBV and HCV infections are the most significant, with viral hepatitis (HBV and HCV infections) accounting for 90% and 40% of liver cancer incidence in developing and developed countries, respectively.

To date, few studies have been conducted to assess the factors leading to liver cancer in HCV patients. At present, HCV RNA, cirrhosis and HCV genotype are thought to affect the occurrence of HCV-related liver cancer, but these factors have not been conclusively proven. At present, 180 million people are chronically infected with HCV, which has been reported to cause more than 350,000 deaths annually [4]. Epidemiological studies also indicate that HCV is associated with a number of extrahepatic manifestations including insulin resistance, type 2 diabetes mellitus, glomerulopathies, oral manifestations and others[5-7].

It has been demonstrated that 55% to 85% of new HCV-infected patients will develop chronic hepatitis C, and 20% to 30% of chronically ill patients will develop cirrhosis and liver failure [8]. Over the course of 30 years, 1% to 3% of patients with HCV without cirrhosis will eventually develop HCC [9,10]. Studies have shown that one-third of HCC cases are caused by hepatitis C[11]. At present, there are three major known mechanisms for HCV-induced HCC: direct pathways involving HCV core proteins, indirect pathways caused by oxidative stress and steatosis, and microRNA (miRNA)-related pathways [12]. Previous studies have shown that while biological signaling systems are complex, the analysis of linear pathways can still provide valuable insights[13].

In this study, we integrated multiple resources, including KEGG pathways, known disease genes, miRNAs and differentially expressed genes, to identify regulatory pathways and key regulators in HCV and HCC from curated trans factor and miRNA regulatory networks. This analysis revealed that *EZH2* and *hsa-miR-155-5p* are critical genes in the development of hepatocellular carcinoma. These genes and pathways may be important biomarkers for predicting HCV-induced HCC. The workflow of our study is shown in Fig. 1.

Materials and Methods

Construction of human Transcription factor and miRNA regulatory networks

The human Transcription factor (TF) and miRNA regulatory networks were built by integrating miRTarBase, TarBase, TRANSFAC and TransmiR[14-16]. These four databases include curated interactions among human TFs, miRNAs, and target genes as well as standardization of gene and miRNA names within the regulatory networks using data from NCBI and miRbase databases. Additionally, all regulatory relationships within the regulatory network were literature-supported. In total, there were 460 TFs, 2,434 miRNAs, 13,898 target genes and 98,894 edges in the regulatory network.

Known HCC and HCV-associated genes and miRNAs

DisGeNET, a discovery platform containing one of the largest publicly available collections of genes and variants associated with human diseases, was utilized to identify two disease-associated genes [17]. Two disease-associated miRNAs were collected from the miR2Disease[18] and HMDD [19] , which are curated databases containing experimental evidence for human microRNA (miRNA) and disease associations. We also utilized genes in the KEGG pathways associated with HCC (168) or HCV (155). We included 30 known HCC-associated genes in DisGeNET and 463 known HCC-associated miRNAs from either miR2Disease or HMDD. Finally, 18 known HCV-associated genes in DisGeNET and 100 known HCV-associated miRNAs in either miR2Disease or HMDD were used for network analysis.

Disease-related network construction

For the disease-related network construction, the closer the nodes in the network to the known disease genes, the more likely they are disease-associated [20]. In order to construct a more closely related subnet, we selected nodes directly connected to the known disease-associated genes in the background network to build an HCC and HCV-related network. In total, there were 409 TFs, 2,300 miRNAs, 10,697 target gene and 48423 edges in this regulatory network.

Differentially expressed genes in the three datasets

The normalized mRNA expression profiles of HCC (TCGA), HCV (GSE15387) and HCV-related HCC (GSE44074) were downloaded from the Gene Expression Omnibus (GEO) database [21] and The Cancer Genome Atlas (TCGA) database [22]. There were 374 HCC samples and 50 normal samples in the TCGA data set, 35 HCV-related HCC samples and 37 HCC samples in the GSE44074, as well as 60 HCV samples and 60 normal samples in the GSE15387. For mRNA expression data, probe sets were mapped to Entrez Gene IDs. When multiple probes corresponded to the same gene, the mean expression value of these probes was used to represent the gene expression level. We obtained 2, 3, and 4 differentially expressed genes at the p -values of less than 0.05 by using edgeR (TCGA data) and SAM (GEO data) in each of the three data sets.

Identification of the subnetworks for each dataset

To construct subnetworks for each dataset, we extracted differentially expressed genes and their neighbor genes from the disease-related network. The regulatory relationships between these genes and miRNAs constituted a core regulatory subnetwork at multiple stages of disease development. We identified 3 subnetworks, which we termed the HCC subnetwork, HCC-HCV subnetwork and HCV subnetwork.

Extraction of candidate risk regulatory pathways

Using the BFS algorithm to extract risk regulatory pathways from the three subnetworks, we identified all the pathways in the network from the nodes indegree 0 to outdegree 0, and pathways with a length greater than 2 were regarded as the candidate risk pathways.

Prediction of key regulators

Gene expression varies in different tissues and during different diseases. Some genes are expressed at a specific stage of a given disease, while some genes continue to play a role throughout the process. We analyzed all the pathways in the three subnetworks to identify the most critical pathways in each network by examining highly shared genes. We propose a KP score to evaluate key pathways, which is calculated as follows:

Where denotes the number of nodes on a pathway within a subnetwork, denotes the number of intersection nodes between pathway and subnetwork , denotes the length of the longest pathway within the subnetwork that satisfies the conditions, denotes location weight score of the intersection gene within the pathway, denotes whether the gene at this position is an intersection gene, if yes, then the value of is 1, if not, the value of is 0, upstream genes get higher scores.

Survival analysis

In this study, we constructed three subnetworks for HCC, HCV samples and normal samples, and identified key pathways from the subnetworks. We next investigated whether the key regulators could distinguish HCC patients with good or poor outcomes. From these data, we obtained TCGA HCC dataset mRNA expression, miRNA expression and clinical information. Next, we used the K-means method (K=2) to cluster all patients into two groups based on the mRNA and miRNA expression. Finally, Kaplan–Meier curve and log-rank tests were used to evaluate the difference in overall survival time between the two groups of patients.

Results and Discussion

Three diseases-related subnetworks

We used three differentially expressed genes to construct three disaease-related subnetworks based on the disease background network, which contained 409 TFs, 2300 miRNAs, 10,697 target gene and 48,423 edges. We then mapped the 247, 237, and 103 differentially expressed genes obtained from the three disease-related differential expression datasets to the background network to obtain three subnetworks. The HCC-related TF-miRNA regulatory subnetwork included 228 edges and 169 nodes, including 22 TFs, 95 miRNAs, and 52 target genes. The HCC-related TF-miRNA regulatory subnetwork included 911 edges and 464 nodes including 46 TFs, 236 miRNAs, and 182 target genes. The HCC-HCV-related TF-miRNA regulatory subnetwork included 513 edges and 307 nodes, including 29 TFs, 157 miRNAs, and 121 target genes (Fig. S1).

Gene ontology (GO) and KEGG functional enrichment analyses were performed to identify the significantly enriched biological processes and pathways in the three subnetworks using DAVID [23]online tools to perform enrichment analysis. The significantly enriched results (FDR<0.05) are shown in Fig. 2. We found that the significantly enriched biological processes included cell cycle, response to organic substance, positive regulation of transcription from RNA polymerase II promoter, and positive regulation of RNA metabolic process. We also found significantly enriched KEGG pathways, such as cell cycle, p53 signaling pathway, pathways in cancer, metabolism of xenobiotics by cytochrome P450, MAPK signaling pathway and Wnt

signaling pathway. Many of these pathways have previously been implicated in HCC and HCV diseases [24-27].

The risk regulatory pathways and key regulators

The BFS method was used to traverse three subnetworks to obtain all pathways in the network with an in-degree of 0 to out-degree of 0, with pathway lengths required to be longer than two nodes. The HCC subnetwork obtained 5,284,069 pathways, the HCC-HCV subnetwork contained 929 pathways, and the HCV subnetwork contained 235 pathways. Each pathway contained several subnetworks, and elucidating key regulators that play important roles in the development of the disease requires identifying the most important of these. To this end, we used KP scores to screen the 5 highest scoring pathways in all the subnetworks (Table 1). All the regulatory pathways were integrated to obtain a network of HCV and HCC processes. From this network, we found that HCV-related genes were mainly enriched in the upstream nodes of the network (green background in Fig. 3), while the genes affecting HCC and HCV were mainly enriched in the middle regions of the network (yellow background in Fig. 3). Finally, genes related to HCC appeared in the downstream regions of the network (violet background in Fig. 3). The network structure also reflected the role of inflammation in carcinogenesis, as many genes associated with inflammatory factors linked nodes in the HCC and HCV networks. To test whether abnormal expression of some core genes at the center of the network affected patient outcome, we examined *hsa-miR-155-5p* , *FOXM1* ,*EZH2* more closely.

EZH2 and *hsa-miR-155-5p* are key regulators

We further analyzed the core genes in the network, and found that*FOXM1* , *EZH2* , *E2F1* and *hsa-miR-93-5p* were significantly correlated with the occurrence of HCC in HCV patients (Fig. S2). Out of these genes, *EZH2* was the most downstream and directly regulated the hub node for *hsa-miR-155-5p* within the network (Fig. S3). This indicated that *EZH2* may be an important gene implicated in the transition from HCV to HCC. Other research has found that *EZH2* and *hsa-miR-155-5p* may play important roles in the progression of both HCC and HCV [28-31], which fits with works indicating that *hsa-miR-155-5p* participates in HCV-induced HCC processes [32,33]. High expression of*EZH2* and *hsa-miR-155-5p* has also been shown to correlate with the severity of HCC [34,35]. Our network analysis indicated that *hsa-miR-155-5p* not only plays a key regulatory role in HCC, but also plays a role in hepatitis-induced liver cancer. Research investigating the effects of controlling the expression of*EZH2* and *hsa-miR-155-5p* in HCV patients may yield new treatment options.

In order to study the correlation of *EZH2* and*hsa-miR-155-5p* expression with HCC survival rate, we compared the expression of these two genes in normal samples versus disease samples. Interestingly, we found that both *EZH2* and *hsa-miR-155-5p*were expressed significantly higher in tumor samples compared with normal tissue. Between the two, we found that *EZH2* was strongly correlated with the survival of patients (Fig. 4), indicating that it may be important for early diagnosis and risk prediction.

Conclusions

It is currently thought that a number of different factors influence HCV-induced HCC. However, due to the lack of appropriate models or data, it is difficult to determine the specific role of HCV in the malignant transformation of liver cells. In order to identify and characterize these mechanisms, researchers have conducted genomic, transcriptomic and epigenomic studies. These studies have revealed gene mutations and gene expression changes that play a role in the development of hepatitis-induced liver cancer. Genomic research has found that long-term hepatitis virus infection causes significant damage, even after eradication of hepatitis virus. Our transcriptomic data indicated that abnormal expression of certain genes and miRNAs is predictive of which patients will later develop HCC. These genes may represent biomarkers which could enable significantly earlier detection of HCC. HCC is not only caused by hepatitis [36]. Therefore, more studies are needed to determine whether genes correlated with HCV-induced cancer are also correlated with liver cancer caused by other factors.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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Figure Legends

Fig. 1 Illustration of the framework for mining regulatory pathways and key regulators by constructing HCV-induced HCC networks.

Fig. 2 A, B and C. The HCC, HCC-HCV and HCV-related TF-miRNA regulatory network GO and KEGG functional enrichment analysis. The numbers represent gene counts for each pathway/GO term within the network. The Q-value represents the Bonferroni-corrected p-value in gene enrichment analysis. The rich factor represents the ratio of the number of genes in the subnetworks to the total number of genes in the pathways/GO terms. (A) Disease-related background network. (B) HCC-related TF-miRNA regulatory subnetwork. (C) HCC-HCV-related TF-miRNA regulatory subnetwork. (D) HCV-related TF-miRNA regulatory subnetwork.

Fig. 3 The key TF-miRNA regulatory network. The orange nodes represent target genes, the blue nodes represent TFs, and the green nodes represent miRNAs. Different border colors represent the gene source of different subnetworks.

Fig. 4 *EZH2* gene differential expression significantly correlated with the overall survival of HCC patients.

Table 1 The KP score of predicted pathways from the three disease-associated subnetworks.

Type	Pathway
HCC	NR4A1-E2F1-hsa-miR-93-5p-EZH2-hsa-miR-155-5p-E2F2-hsa-miR-92a-3p-CDK1
HCC	NR4A1-E2F1-hsa-miR-93-5p-EZH2-hsa-miR-155-5p-E2F2-hsa-miR-92a-3p-HIST1H2AM
HCC	hsa-miR-26b-5p-FOXM1-hsa-miR-200b-3p-EZH2-hsa-miR-155-5p-CDKN2A-hsa-miR-141-3p-CDC25C
HCC	STAT3-FOXM1-hsa-miR-200a-3p-EZH2-hsa-miR-155-5p-PLK1-hsa-miR-141-3p-CDC25C
HCC	STAT3-FOXM1-hsa-miR-200a-3p-EZH2-hsa-miR-155-5p-PLK1-hsa-miR-141-3p-CDC25C
HCC-HCV	MYC-FOXM1-hsa-miR-200b-3p-MYB-hsa-miR-155-5p-CHD8
HCC-HCV	MYC-FOXM1-hsa-miR-200a-3p-MYB-hsa-miR-155-5p-RAP1B
HCC-HCV	MYC-FOXM1-hsa-miR-200b-3p-MYB-hsa-miR-155-5p-RAP1B
HCC-HCV	MYC-FOXM1-hsa-miR-200a-3p-MYB-hsa-miR-155-5p-SLC39A14
HCV	hsa-miR-155-5p-CBFB-APC
HCV	hsa-miR-155-5p-CBFB-hsa-miR-221-3p-BBC3
HCV	hsa-miR-155-5p-CBFB-hsa-miR-221-3p-GJA1
HCV	hsa-miR-124-3p-NR4A1-E2F1
HCV	hsa-miR-93-5p-NR4A1-E2F1

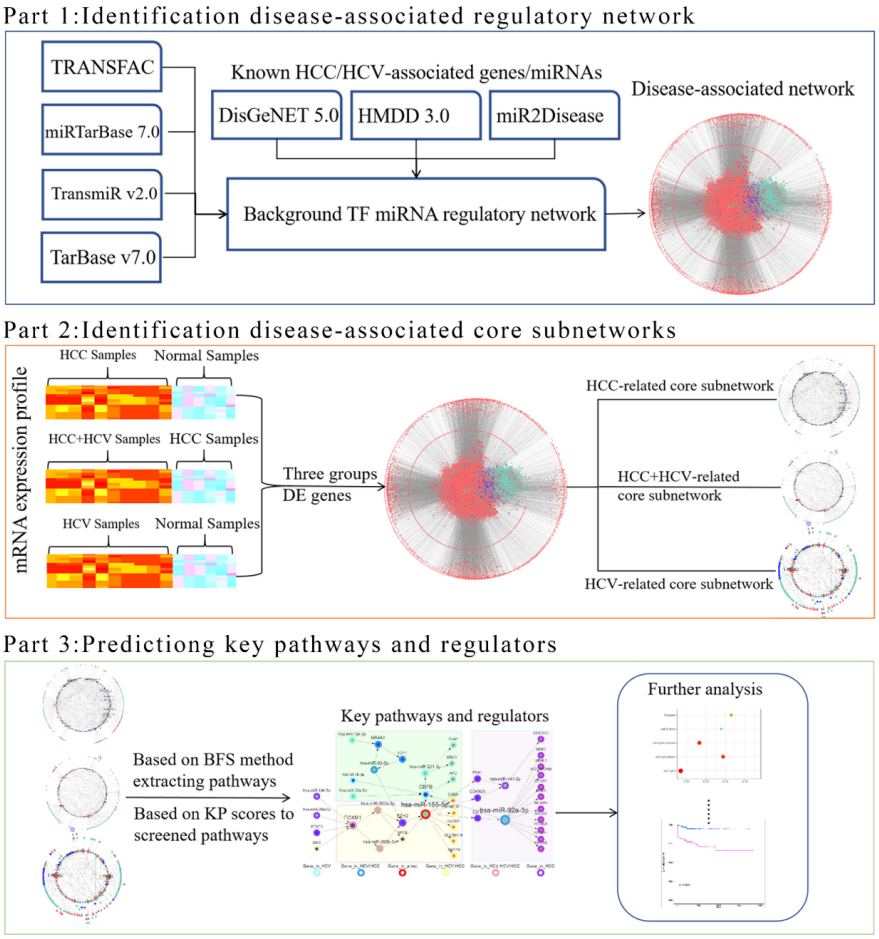
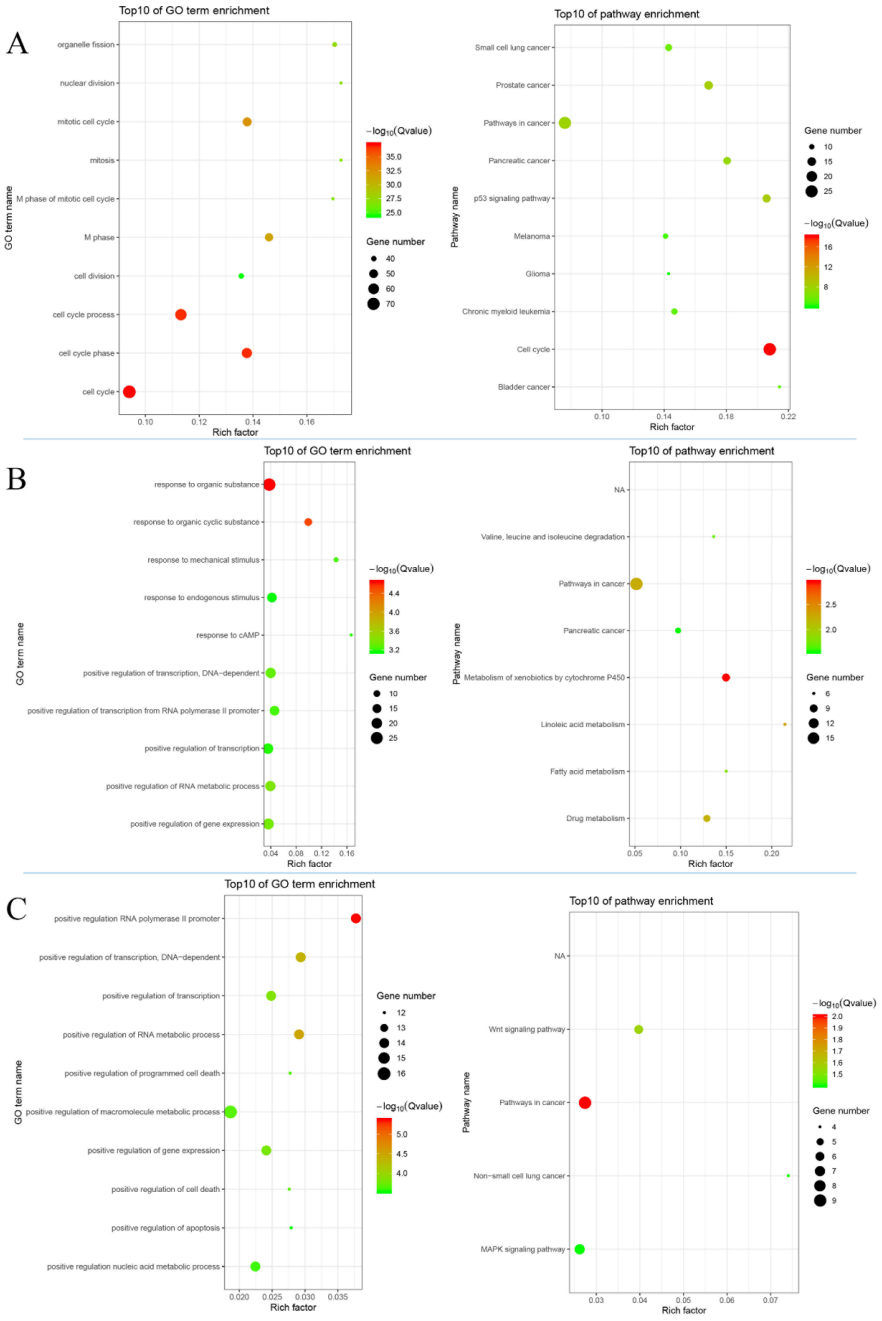


Figure 1



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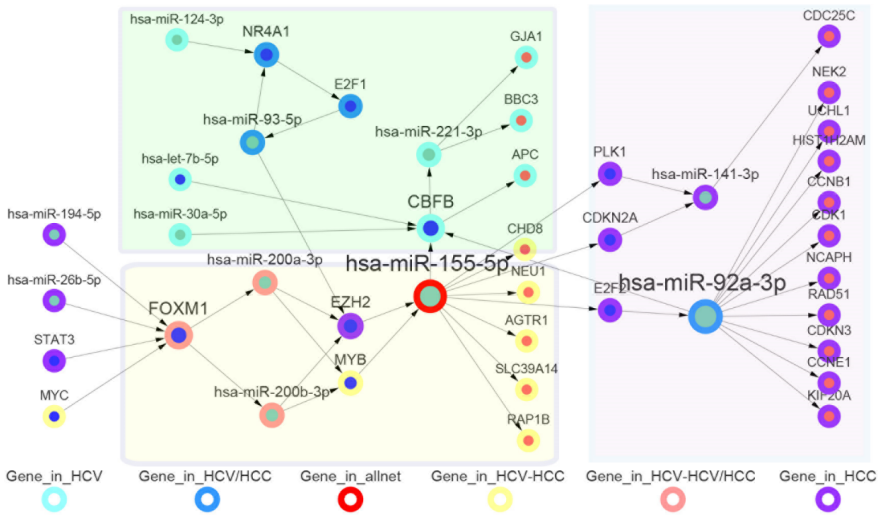

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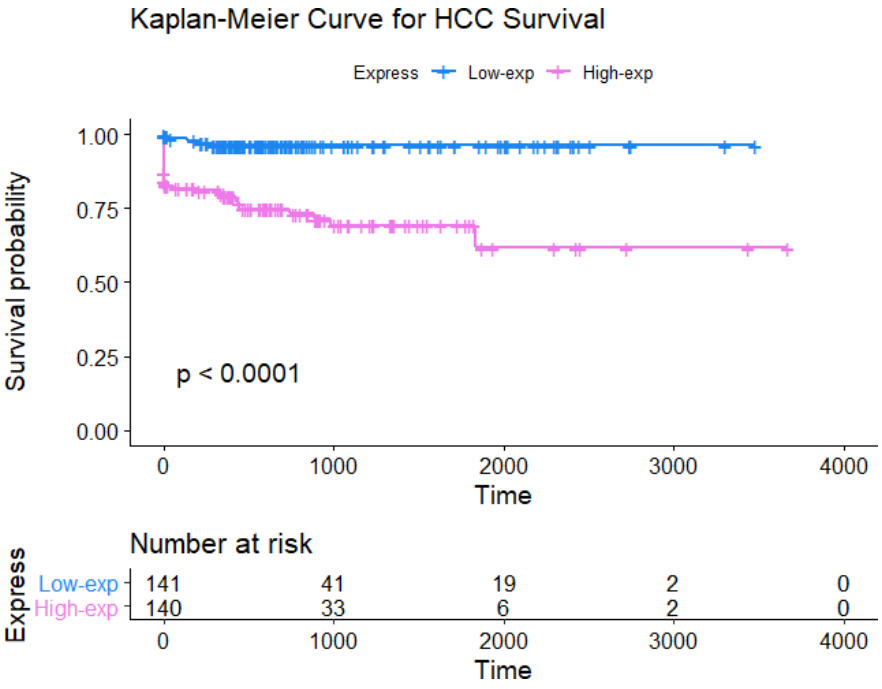

Figure 4