Comprehensive Identification of Trem2 as a Key Factor in Promoting the Progression of Hepatocellular Carcinoma and Predicting Prognosis

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Primary research

Keywords: hepatocellular carcinoma, HCC, CDAHFD60, progression of HCC

Posted Date: September 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-835818/v1

Abstract

Backgrouds

Trem2 is a core member of the Triggering recptor expressed on myeloid cell family, and its role in response to pathological damagewas first discovered in Alzheimer's disease. Recently, the role of Trem2 played in tumor progression has been highlighted. However, the specific role and related mechanisms of trem2 in HCC (hepatocellular carcinoma) are still ambiguous.

Methods

The 371 HCC data sets in the TCGA cohort combined with the TIMER2.0 online database were used to verify the potential role and prognostic value of trem2 in HCC.TIMER2.0 online database,immunohistology, immunofluorescence, Western Blot and other methods are applied to identify the correlation between Trem2 and HCC patient samples.Animal models of bile duct ligation, intraperitoneal injection of CCL4 and feeding of CDAHFD60 were used to verify the potential mechanism of trem2 to regulate HCC.

Results

Trem2 is significantly overexpressed in HCC and is an independent risk factor predicts the poor prognosis of HCC patients. Trem2 mainly regulates the progression of HCC in response to the mutations of TP53 and CTNNB1, which also suggests that Trem2 plays an important role in tumor pathological metabolism. Since HCC patients often accompanied by different degrees of liver fibrosis, we suspect that Trem2 may promotes the progression of HCC by regulating liver fibrosis.

Conclusion

Our research highlights the status of trem2 in the tandem or independent events of HCC's pathological metabolism, immune checkpoint coordination, and liver fibrosis. We predict that targeting Trem2 combined with immune checkpoint inhibitors may be an effective treatment for HCC with rich immune and metabolic microenvironment.

Introduction

Hepatocellular carcinoma (HCC) is the sixth largest cancer in the world and the fourth leading cause of cancer-related death due to the lack of effective treatments and interventions[2].In high-burden regions such as East Asia and Africa, the main risk factor for liver cancer is HBV infection[3].At present, liver resection, liver transplantation, liver ablation, TACE, and other methods are used lonely or in combination only for the treatment of early HCC, in order to expect a fairly limited disease remission[4].

Although there are some progress in the diagnosis and treatment of liver cancer, the pathogenesis and mechanism of HCC are still unclear, so there is a lack of effective clinical transformation[5].As we all

known, the process of driving HCC progress is a complex and multi-step cascading procedure. The interaction of various factors including viruses, fatty liver, genetic susceptibility, etc. leads to the deterioration of liver cells and initiates the HCC cascade.

At present, the practice of systemic therapy represented by immune checkpoint inhibitors (ICI) and monoclonal antibodies has been successfully carried out in HCC. The combination of atezolizumab (anti-PDL1 antibody) and bevacizumab (anti-VEGF antibody) more than doubled life expectancy and increased the benefits of systemic therapy for HCC patients[6]. Researches have shown that the combination of cabozantinib and nivolumab brings down-stage resectable benefits to patients with advanced HCC[7]. Sorafenib and levantinib are still the first-line treatments for HCC [8]. Nevertheless, the efficacy of systemic therapy and monoclonal antibody therapy still needs to be improved due to the complex immune and metabolic microenvironment of the liver.

In the liver, Trem2 is overexpressed in resident nonparenchymal cell of liver (kupffer cells, hepatic stellate cells) and recruited cells (neutrophils and monocytes) when it responds to many types of liver injury [9, 10].In tumors, the systemic knockout of Trem2 would promote the progression of HCC. Relevant studies also revealed that Trem2 can protect the liver from HCC [11], which is completely different from the effect of targeting myeloid Trem2 on HCC [12]. It may be due to the confounding of Trem2 targeting in this study that is difficult to reveal its specific mechanism. The role of TREM2 in HCC is complicated due to a variety of confounding and even distinct findings of identity reveal.

In our study, we have clearly shown that the expression of Trem2 in HCC is significantly up-regulated by integrating information in public databases. The expression of Trem2 is independent of HBV background, but is significantly related to TP53 and CTNNB1 mutations. The clinical evaluation showed a obvious correlation between Trem2 and T staging and Grade, and the univariate and multivariate Cox analysis identified Trem2 as an independent risk factor. In addition, Trem2 has been shown to play an important role in regulating fatty acid metabolism and other pathways. The TREM2 enriched genes obtained by KEGG analysis including ACSL4 and ADH1A are important participants in fatty acid metabolism and liver cancer progression. Furthermore, many in vivo experiments in mice show that Trem2 is also essential in the progress of liver fibrosis. Liver fibrosis is an important prelude to HCC of different origins, so Trem2 may participate in progress of HCC by regulating fibrosis. Importantly, Trem2 was found to be consistent with the expression of immune checkpoints, which suggests the great potential of Trem2 as a single targeted drug or combination drug.

Methods And Materials

1. Patients’ Samples

Twelve pairs of HCC and adjacent samples came from the Hepatobiliary Center of the First Affiliated Hospital of Nanjing Medical University. The slices of the patients were evaluated by Ishak classification,

and 10 of them had liver cirrhosis. All experiments were conducted in accordance with the requirements of the Ethics Committee of Nanjing Medical University, and the patients signed an informed consent.

2. Acquisition and analysis of microarray data

We download the clinical information and RNA-Seq data of 371 samples (including normal samples and HCC samples) from the public database TCGA (https://portal.gdc.cancer.gov/.), and then use the Limma R package Integrate patient data, analyze trem2 from the perspectives of differential expression analysis of homologous paired tissues, clinical TNM staging and Grade grading, synergy of immune checkpoints, and HCC mutation background through R software

(3.6.3 ,https://www.r-project.org) .

3. The enrichment analysis of GO, KEGG of Trem2

Gene expression and biological characteristics in a high-throughput context are analyzed by GO (Gene Ontology, including three modules of biological process (BP), cell composition (CC) and molecular function (MF))and Kyoto Encyclopedia of Genes and Genomes (KEGG). This experiment is run through the "clusterProfiler" R package for analysing the GO terms of Trem2 and the bubble chart enriched by KEGG, and the data is visualized through the "ggplot2" package.

4. Murine Experiments

CCL4 model: 6–8 weeks male C57 mice were purchesed from Vital River Laboratory Animal Technology Co. Ltd (Beijing,China).were injected intraperitoneally with CCL4 (0.6ul/g body weight,CCL4: olive oil = 1:3) every 3 days, continuously administered for 4w or 8w.

BDL model: Let adult C57 mice receive isoflurane inhalation anesthesia. After opening the abdominal cavity, the common bile duct was ligated with 4 – 0 vicryl Rapide. The sham operation group underwent the same operation except for the steps of common bile duct ligation. The mice were sacrificed 2w or 4w after the operation and the experimental livers were collected.

NASH model: CDAHFD60 has been shown to be a feeding model that rapidly induces fibrosis after hepatic steatosis in mice[1]. CDAHFD60 feed was purchased from Dyets (Dyets Biotech). We killed the mice after 8 weeks of feeding CDAHFD60 and obtained fibrotic liver. All experimental mice were euthanized after the experiment following the recommendations of AVMA.

5. qPCR

After extracting total RNA from the tissue, it is reverse transcribed into cDNA. Then use SYBR Green fluorescent dye to perform real-time fluorescent quantitative polymerase chain reaction, and all expression levels and results of target genes are standardized for GAPDH expression.

6. Immunohistochemistry and immunofluorescence

Collect experimental liver tissues and soak them in 4% paraformaldehyde for 24–72 hours and then slice the fixed tissues after embedding in paraffin. For Confocal Immunoflfluorescence,Cover the embedded sample with 3% BSA uniformly□then add the prepared primary antibody and incubate overnight at 4° C. Drop the overnight samples in the histochemistry kit with the secondary antibody□After the sections were shaken dry, DAPI dye solution was added and incubated□The slides were dried slightly and then mounted with antifluorescence quenching mounting tablets. The slices were observed under a confocal fluorescence microscope (NIKON ECLIPSE C1), and images were collected.

7. data visualization

The data from the TCGA public data set is analyzed in R Studios through the corresponding R package. Hiplot (http://hiplot.com.cn) multi-module data calculation and visualization platform is also used to integrate and analyze data from Trem2 from the TCGA database. Kaplan-Meier method draws survival curves and compares them with log-rank test. $P < 0.05$ is considered to indicate a statistically significant difference.

8. Statistical Analysis

Use R software (3.6.3) to run "survival", "Unicox", "multicox" and other R packages to visually output the analysis results of Trem2 in 371 HCC patients. The Kaplan-Meier curve of 371 HCC patients was drawn. Univariate and multivariate Cox regression analysis were performed using the "survival" R package. Wilcox test was used to analyze the association between Trem2 expression and clinicopathological characteristics ($P < 0.05$). The Kaplan-Meier method was used to draw the survival curve of 371 HCC patients and the log-rank test was used for comparison. The above analysis is considered to indicate a statistical difference in the case of $P < 0.05$.

Results

1. Trem2 is highly expressed in HCC

Screening the expression of Trem2 in all primary tumors through the TIMER2.0 online database (http://timer.cistrome. org/)[13] showed that Trem2 was significantly up-regulated in 17 cancer types including HCC (1A), indicating the importance of Trem2 in tumorigenesis and development. The differential analysis and paired differential analysis of the mRNA expression of trem2 in the 371 HCC cases downloaded from the TCGA database showed that the transcription level of trem2 in the tumor tissue was significantly higher than that in the homologous adjacent normal liver tissue (1B, 1C). Although there are a few transcriptome data showing the relative down-regulation of trem2 in HCC tissues, this may be due to individual differences or heterogeneity of cell promiscuity. Therefore, we verified it in our patient samples. Excluding the pathogenic factors and background, through immunohistochemistry and immunofluorescence examination of 12 HCC patients, we found that the expression of trem2 in HCC was significantly increased (1D, 1E). Transcription examination of HCC tissue and distal tissues of each patient also showed a significant upregulation of Trem2 (data not shown).

2. Trem2 is an independent prognostic factor of HCC and has nothing to do with whether patients have an HBV background.

The COX test was performed on the HCC data of the TCGA cohort to evaluate the impact of various risk factors on the survival time of HCC patients. The results showed that univariate or multivariate cox regression analysis identified Trem2 as an independent prognostic factor affecting the overall survival of HCC patients (p = 0.01, 0.04, 2A, table 1). HCC patients often have an HBV background[14]. Therefore, we need to know whether Trem2 depends on the HBV background to participate in the regulation of HCC process. Wilcox test analysis showed that trem2 does not rely on HBV to participate in the regulation of HCC (P = 0.17, 2B), it means that trem2 may drive the development of HCC by not alone responding to the damage caused by viral DNA.

3. The high expression of Trem2 is related to the clinical stage and grade of HCC and indicates a poor prognosis

Tumor TNM staging and Grade classification are important references for clinical evaluation of tumor progression and prognosis[15]. We checked the internal relationship between trem2 and TNM staging and Grade in HCC through the R software (3.6.3). We found that Trem2 and T staging and Grade have a significant positive correlation (p = 2.1e-13, 1.5e-13, 3A). We then examined the relationship between the expression of trem2 and the prognosis of HCC patients through survival analysis. We found that high expression of trem2 predicts a worse prognosis for HCC patients (p = 0.0086, 3B).

4. Trem2 expression in different HCC mutation backgrounds

The detection of the whole transcriptome combined with the proteome identified TP53, AXIN1, CTNNB1, KEAP1 and RB1 as the genes with the highest mutation tendency in HCC [14]. TP53 is the most common mutant gene in HCC. Not surprisingly, HCC with TP53 mutation has a higher trem2 transcription tendency than WT HCC (P = 3.4E-06, 4A); according to the study of Gao et al.[14], Among patients in the CHCC-HBV cohort, the mutation frequency of AXIN1 was higher than that of the HBV cohort in the TCGA database. The expression of trem2 in HCC patients with AXIN1 mutations screened by TIMER 2.0 was not significantly different from that of the WT group (P = 0.18, 4C). Although the number of samples in the AXIN1 mutation group is small (n = 23/500), the no difference between the groups is more likely to be related to whether the HBV background has no significant effect on the expression of Trem2; CTNNB1 mutations rely on various metabolic pathways to drive HCC progression, such as Drug metabolism, glycolysis/gluconeogenesis and amino acid metabolism. Unlike the TP53 mutation, the expression of Trem2 was significantly down-regulated in patients with CTNNB1 mutation (p = 1.8e-5, 4A). This prompted us to explore the potential role of trem2 in regulating liver metabolism. We have used cluster Profiler R package confirmed through Go functional enrichment and KEGG (Kyoto Encyclopedia of Genes

and Genomes) enrichment analysis that the expression of trem2 is indeed negatively correlated with pathways including drug metabolism and glycolysis/gluconeogenesis (4A), which reflects trem2 expression has a high compliance that is strongly negatively correlated with CTNNB1 mutation. In addition, trem2 is also most negatively related to retinol metabolism and chemical carcinogenesis (4C). The pathways for trem2 up-regulation include P53, FC gamma R and other pathways (4D); Keap1 and RB1 mutations also have no significant effect on the expression of trem2 (p = 0.49,0.24,4A). The volcano map shows that 2395 genes including trem2 are up-regulated and 463 genes are down-regulated in HCC in the TCGA database (4B).

5. The correlation between Trem2 in HCC and multiple immune checkpoints

TIMER2.0 was used to measure trem2 and PDL1 (CD274, Spearman's rho = 0.268, p = 1.57e-07), VEGFA (Spearman's rho = 0.152, p = 3.32e-03), CD276 (Spearman's rho = 0.488, p = 1.36e-23), TIGIT (Spearman's rho = 0.453, p = 3.87e-20), CTLA4 (Spearman's rho = 0.507, p = 1.23e-25), SIRPa (Spearman's rho = 0.342, p = 1.37e-11) and other immune checkpoints were positively correlated (5A-F). The currently approved clinical systemic immune strategies for the treatment of HCC are not very effective. Although current clinical trials show that when anti-PD-1 or anti-PD-L1 drugs are used in combination with anti-CTLA4, the response rate and response duration are satisfactory to some extent[16], we still need to explore new methods to enhance the immunosuppressive efficacy of HCC therapy. Therefore, our analysis shows that targeting trem2 or combined targeting may be a promising strategy in HCC.

6. GO and KEGG identified Trem2 is related to fatty acid metabolism

A large study showed that genes related to FA biosynthesis in HCC tissues were significantly up-regulated[17]. In fact, the generation of FA shows considerable potential in driving the progress of HCC. KEGG identified fatty acid metabolism (ES: -0.87) as the functional pathway with the highest negative correlation enrichment index for trem2. Among the identified fatty acid metabolism pathways, genes with high negative correlation ES with Trem2 mainly include ACSL (ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6) family and ACAT family (ACAT1, ACAT2) (Table 2). The ACSL family exists in mammals and contributes to anabolic lipid biosynthesis and catabolic fatty acid oxidation. Most current research results indicate that the ACSL family has a cancer-promoting effect [18]. ACLY (ATP citrate lyase) is an enzyme involved in lipid synthesis. Multiple methods have proven that ACLY can be used as a prognostic marker of HCC [19]. Increased expression and phosphorylation of ACLY, ACSL3, and ACSL4 in HCC have been shown to activate lipid biosynthesis in HCC [14]. In HCC, Trem2 has a positive correlation with the expression of ACLY on the one hand (rho = 0.283, p = 3.04e-08, 6B), on the other hand, it has a negative correlation with the expression of ACSL1. Surprisingly, the expressions of ACSL3 and ACSL4 and trem2 were positively correlated in HCC after purity adjustment by TIMER 2.0 database, this is interesting. After further exploration, ADH1A is also a highly negatively correlated ES gene of trem2 enriched in the fatty acid metabolism pathway. It was previously found to be down-regulated in different subtypes of HBV-HCC and

indicated a poor prognosis, and the worst prognosis S-Pf The expression of ADH1A in proliferative HBV-HCC was the lowest[14]. Trem2 transcription can significantly down-regulate the expression of ADH1A (6E, Table 2). HCCs with high ADH1A expression have fewer TP53 mutations, which in turn leads us to think about the internal connection between trem2, ADH1A and TP53 mutations. PPAR-a (rho=-0.126, p = 1.54e-02, 6A) and ACAT1 (rho=-0.202, p = 1.63e-04, 6D) have also been shown to have a significant negative correlation with trem2. Analyze the protein network of Trem2 by STRING (string-db.org). It is already known that Trem2 is mainly enriched with TYROBP and SYK (6F). Then we customized analysis of fatty acid pathway-related genes enriched in trem2 screened from KEGG in STRING. Although trem2 is currently unable to be cross-linked into the fatty acid metabolism grid due to the research blank, its enriched related genes have constructed a complete fatty acid metabolism grid. In general, for HCC RNA-Seq, the role of trem2 in fatty acid metabolism is contradictory. This may be due to the identity confounding of whole transcriptome sequencing, or it may be due to Trem2 foreshadowing huge cellular heterogeneity. Regardless, these results prompt us to explore the powerful potential of Trem2 in fatty acid metabolism and metabolism-driven HCC.

7.TREM2 may regulate the progression of HCC by promoting fibrosis

KEGG enrichment analysis proved that Trem2 has a positive correlation with fibrosis regulatory factors Col1A1/2 and MMP9 (7A)[20]. PPARα has been shown to improve fatty degeneration, inflammation and fibrosis of NAFLD, Therefore, Trem2 negatively regulates PPARa to affect liver lipid metabolism and fibrosis is one of the possible mechanisms[21]. Its indicates that Trem2 may be involved in regulating the occurrence of fibrosis. Fibrosis is often the common destination of most hepatitis B infections and NAFLD progression[22, 23]. Detect the expression of Trem2 and αSMA in human fibrosis (quantified by Ishak staging) and HCC with cirrhosis (7B-C). Trem2 showed that the expression level gradually increased with the progress of fibrosis and HCC, and it co-localized strongly with HSC of αSMA mark. This shows that Trem2 is a positive factor in promoting fibrosis. Subsequently, we induced fibrosis during CCL4, BDL, CDAHFD60 models to detected the high expression of Trem2 (7D-F).

Discussion

It is estimated that by 2030, HCC will become the third leading cause of cancer-related deaths worldwide[24]. Although the current large-scale vaccination of HBV vaccines in East Asian countries has led to the relief of the disease burden of HBV-related HCC, the large number of chronic HBV hepatitis progressing to liver cirrhosis and hepatocellular carcinoma HCC is still an important clinical trilogy in the Asia-Pacific region [25]. Globally, the incidence of HCC developed by NAFLD and NASH is gradually increasing, which is derived from a new disease pattern. It is similar to HCC developed by HBV in that many patients will experience liver fibrosis[26]. Extensive liver fibrosis is the main feature of liver cirrhosis and has become one of the important reasons that threaten the safety and health of humans worldwide. Liver fibrosis is positively correlated with the high incidence of HCC, and is an important independent risk factor for the poor prognosis of patients with HCC and chronic liver diseases[27]. However, no effective

anti-fibrosis therapy has been born yet. In our study, we identified trem2 as a factor positively related to the formation of CAF (cancer associated-fibroblast) and the induction of the expression of fibrous collagen factor Col1a1/2 and αSMA through the Timer2.0 online database. Through the practice of liver fibrosis model induced by various mechanisms of C57 mice, we have proved that Trem2 is an important role in the formation of liver fibrosis. This may be one of the mechanisms by which Trem2 regulates the progress of HCC.

In recent years, the role of Trem2 in the progression of a variety of tumors has become the focus of academic research. A single-cell sequencing based on tumor-associated macrophages showed that Trem2 has the ability to survive and prognosis for triple-negative breast cancer independent of other myeloid genes [12]. Not only that, the expression level of Trem2 predicts the poor prognosis of a variety of solid tumors. Targeting myeloid Trem2 has been shown to improve the myeloid infiltration of the tumor microenvironment and enhance the efficacy of PD-1 [12]. It is particularly noteworthy that a blockbuster study showed that Trem2 has abnormally rich infiltration in the liver metastasized from solid tumors [12]. In a study of scRNA-Seq for cirrhosis, a group of scar-related TREM2 + CD9 + macrophages in fibrotic liver was defined, which promotes fibrosis and expands widely in fibrotic liver [28]. NASH diet in mice induces Trem2 and CD9 expression and is associated with liver fibrosis [29]. However, several experiments on Trem2-/- have verified the protective effect of Trem2 on the liver. Therefore, the cell targeting of different therapies indicates the huge heterogeneity of the role of Trem2, which also makes the research on Trem2 complicated and mysterious. At present, Trem2 expressed by different cell sources has not been explored in HCC. Therefore, we have exerted multiple analysis modules such as TCGA cohort, single-factor and multi-factor COX analysis, mutation correlation, survival analysis, GO and KEGG enrichment analysis, etc.to prove that Trem2 is an independent factor predicting poor prognosis of HCC.

At present, the successful clinical application of immunosuppressants such as PD-1 and CTLA-4 heralds the rise of immune checkpoint inhibitor therapy. Especially antibodies targeting the programmed cell death-1 (PD-1)/programmed cell death ligand-1 (PD-L1) pathway, represent cancer drugs in the past decade A major breakthrough in development[16]. Thousands of clinical trials based on immune checkpoint inhibitor therapy are in full swing around the world. It needs to be recognized that the immunosuppressive myeloid cells infiltrated by the tumor microenvironment, such as MDSC and TAM, are important factors that exacerbate T cell immunosuppression[30]. Therefore, how to target myeloid suppressor cells to regulate the progression of HCC is a fascinating area. In HCC, Trem2 has a significant correlation with immune checkpoints of HCC that have been clinically proven or promising. The development of targeting Trem2 in combination with other immunosuppressive agents is attractive enough. Combined with its status as an independent prognostic factor for HCC, it suggests that trem2 may be a dawn for HCC immunotherapy to break through the long night.

In general, we explored the identity and plasticity of Trem2 involved in the development of HCC from multiple dimensions such as HCC's clinical indicators, immune infiltration, immune checkpoints, gene mutations, and metabolic pathways. The role of Trem2 in promoting the progress of HCC should be clear, but the mechanism must be complicated. Although HCC may affect the occurrence of HCC from multiple

perspectives such as affecting fatty acid metabolism, the role of Trem2 in regulating liver fibrosis may be the intersection of multiple regulatory pathways.

Conclusion

Our study identified Trem2 as an important factor that can predict the risk and prognosis of HCC. Nevertheless, further research is needed to explore the cell targeting and heterogeneity of trem2's regulatory role, and to explore its mechanism of action.

Abbreviations

HCC: Hepatocellular carcinoma

TCGA:The Cancer Genome Atlas

TME:Tumor microenvironment

Trem2: Triggering recptor expressed on myeloid cell 2

GSEA:Gene set enrichment analysis

GO:Gene Ontology

KEGG:Kyoto Encyclopedia of Genome and Genome

BP:Biological process

CC:Cellular component

MF:Molecular functional

PD-1:Programmed cell death protein 1

PD-L1:Programmed death 1 ligand 1

ACLY:ATP citrate lyase

CI:confidence interval

Declarations

Fundings

This study was supported by grants from the National Natural Science Foundation of China (81870443, 81273261), and Shenzhen Foundation of Science and Technology (grant number

JCYJ20170817172116272), Sanming Project of Medicine in Shenzhen (SZSM201812079).

Contributions

Conceptualization, HS and WZY; methodology, YWJ; validation, JCY, JCY, CXJ; data curation, LXD, XN and ZY; writing—original draft preparation, HS, WZY and YWJ; writing—review and editing, HS, WZY and YWJ; supervision, PLY; project administration, PLY and NY; funding acquisition, PLY and NY. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures performed in studies involving human participants and murine experiments were in accordance with the ethical standards of the Research Ethics Committee of Ethics Committee of Nanjing Medical University.

Consent for publication

The authors agree for publication.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

All data available.

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Tables

Table 1

	Univariate Analysis		Multiivariate Analysis	
	HR (HR.95L-HR.95H)	P value	HR	P value
age	1.01(0.99-1.02)	0.59	1.01(0.99-1.03)	0.47
gender	0.78(0.49-1.25)	0.30	1.02(0.61-1.71)	0.93
Grade	1.02(0.75-1.39)	0.91	1.07(0.77-1.50)	0.69
Stage	1.86(1.46-2.39)	0.00	1.17(0.44-3.09)	0.76
T	1.80 (1.43-2.27)	0.00	1.55(0.64-3.73)	0.33
M	3.85 (1.21-12.28)	0.023	1.07(0.28-4.04)	0.92
N	2.02 (0.49-8.28)	0.33	1.57(0.23-10.90)	0.65
Trem2	1.29 (1.08-1.54)	0.01	1.21(1.01-1.46)	0.04

Table 2

Due to technical limitations, Table 2 is only available as a download in the Supplemental Files section.

Figures

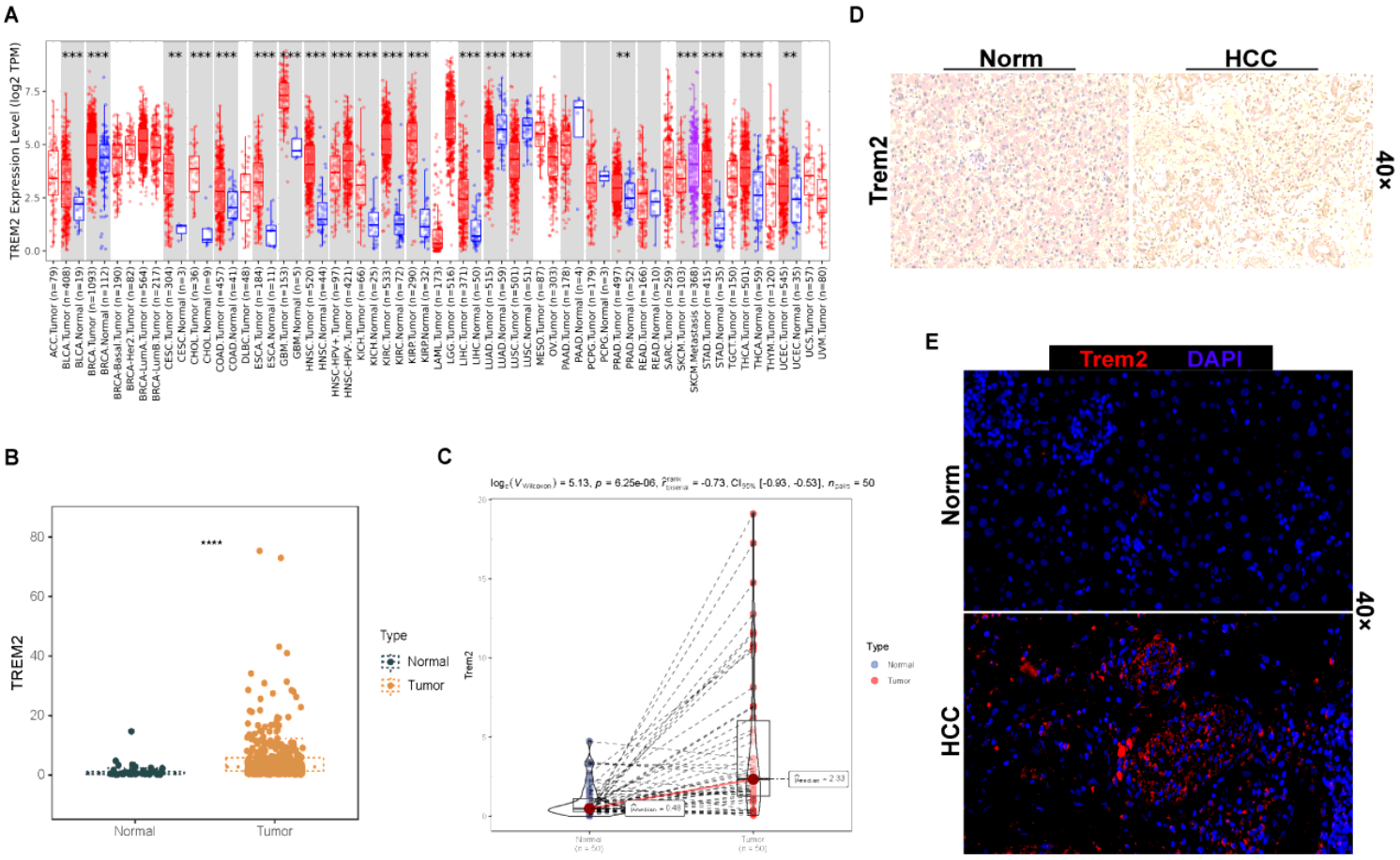

Figure 1

Caption not included with this version.

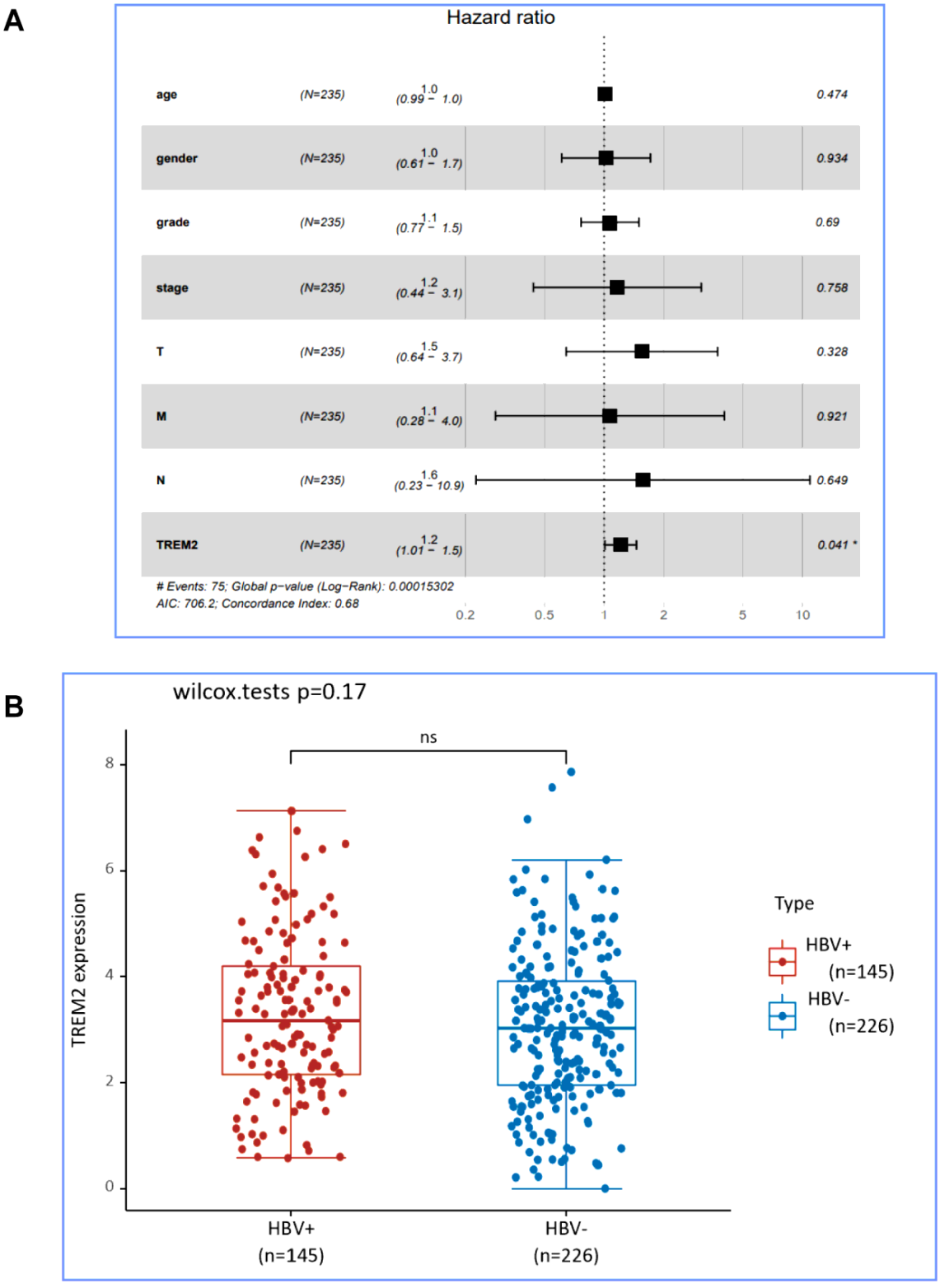

Figure 2

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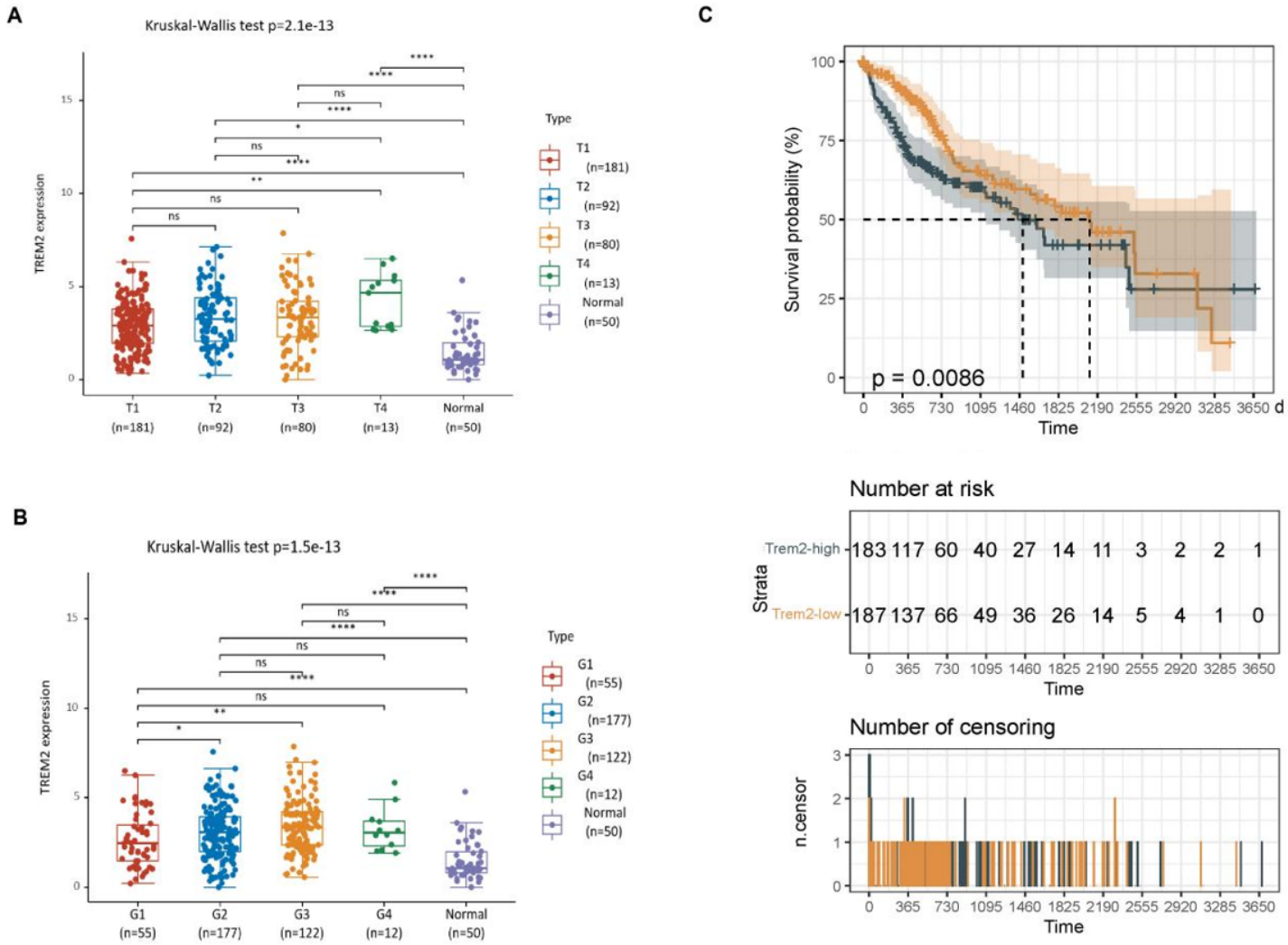

Figure 3

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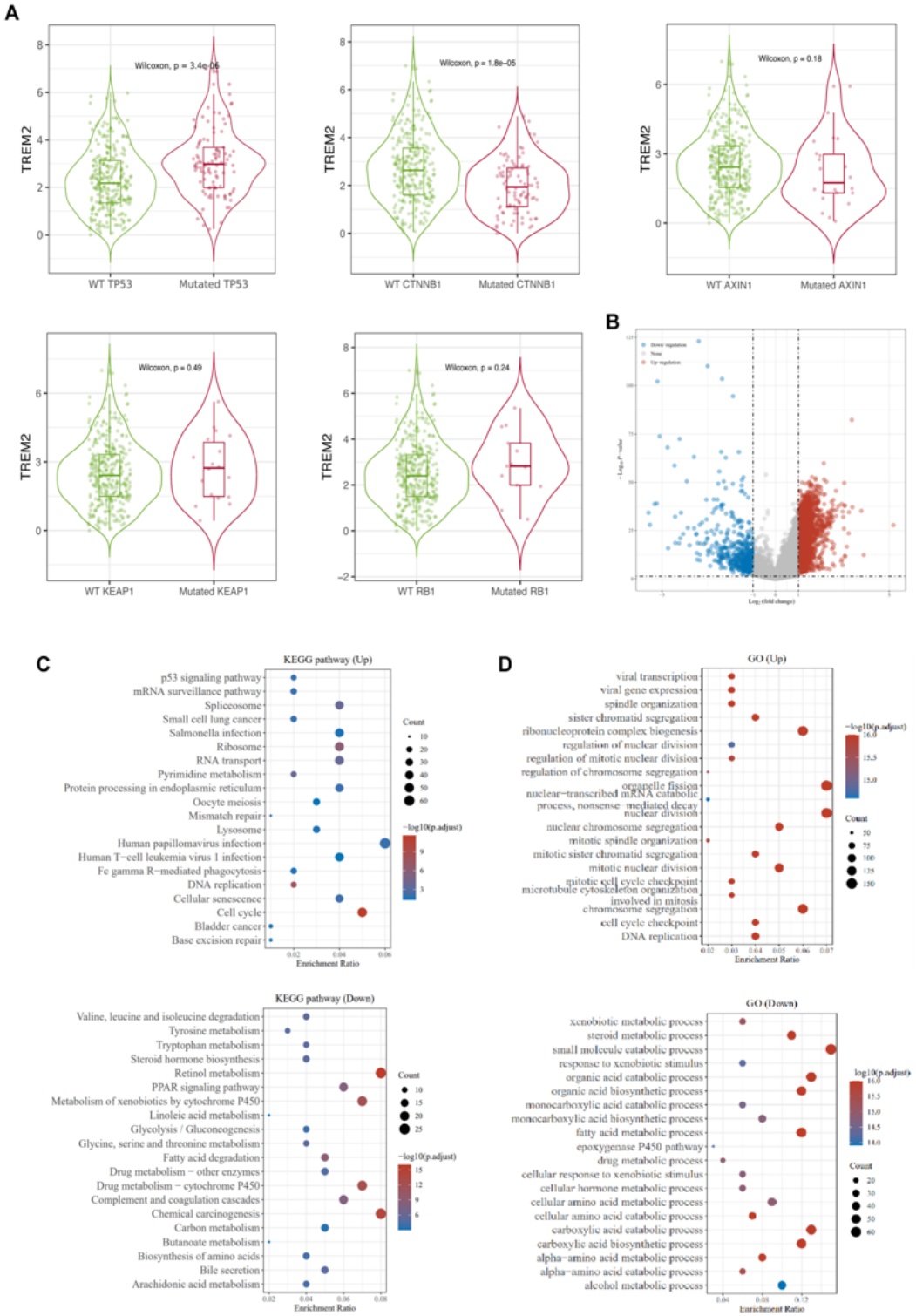

Figure 4

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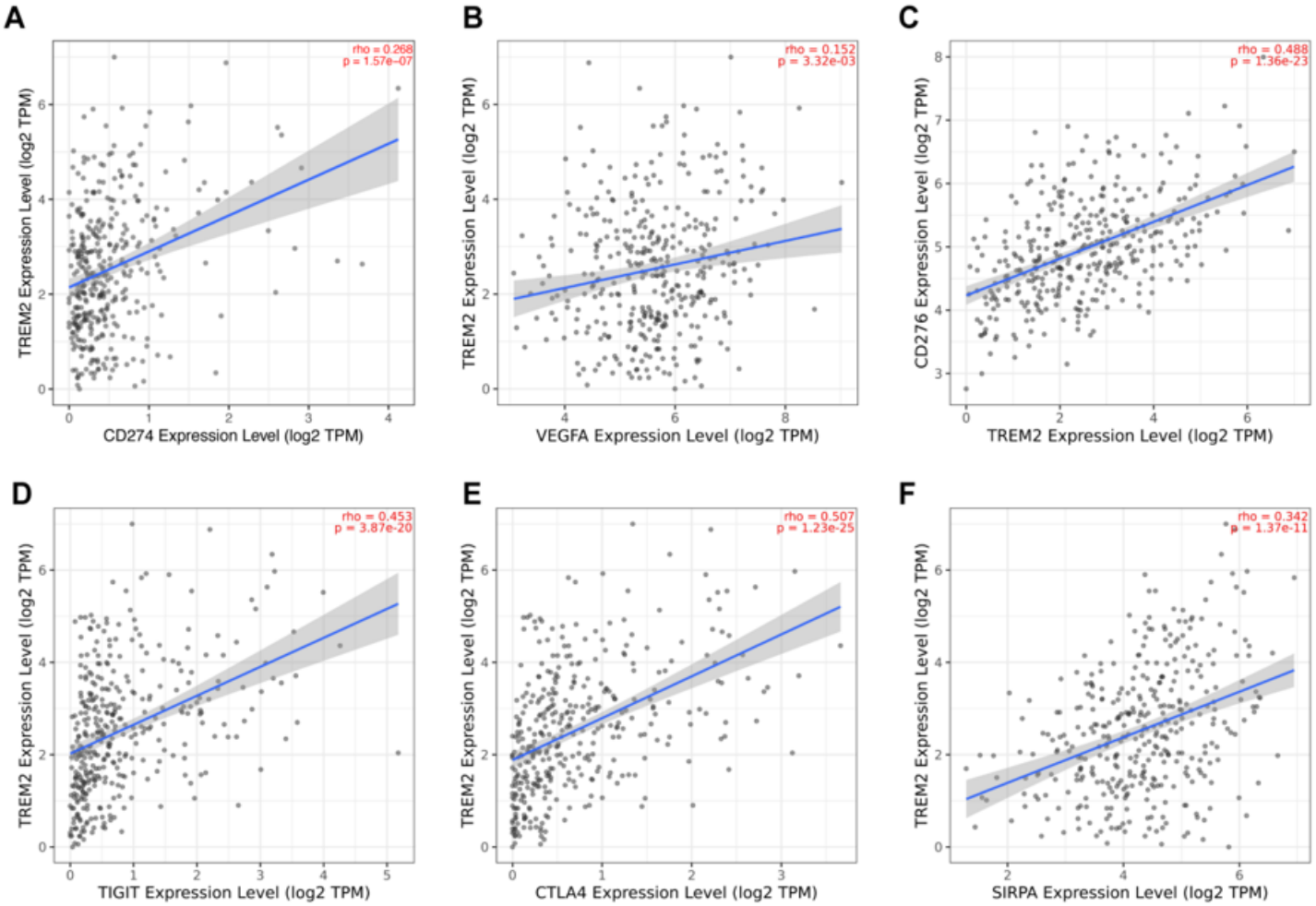

Figure 5

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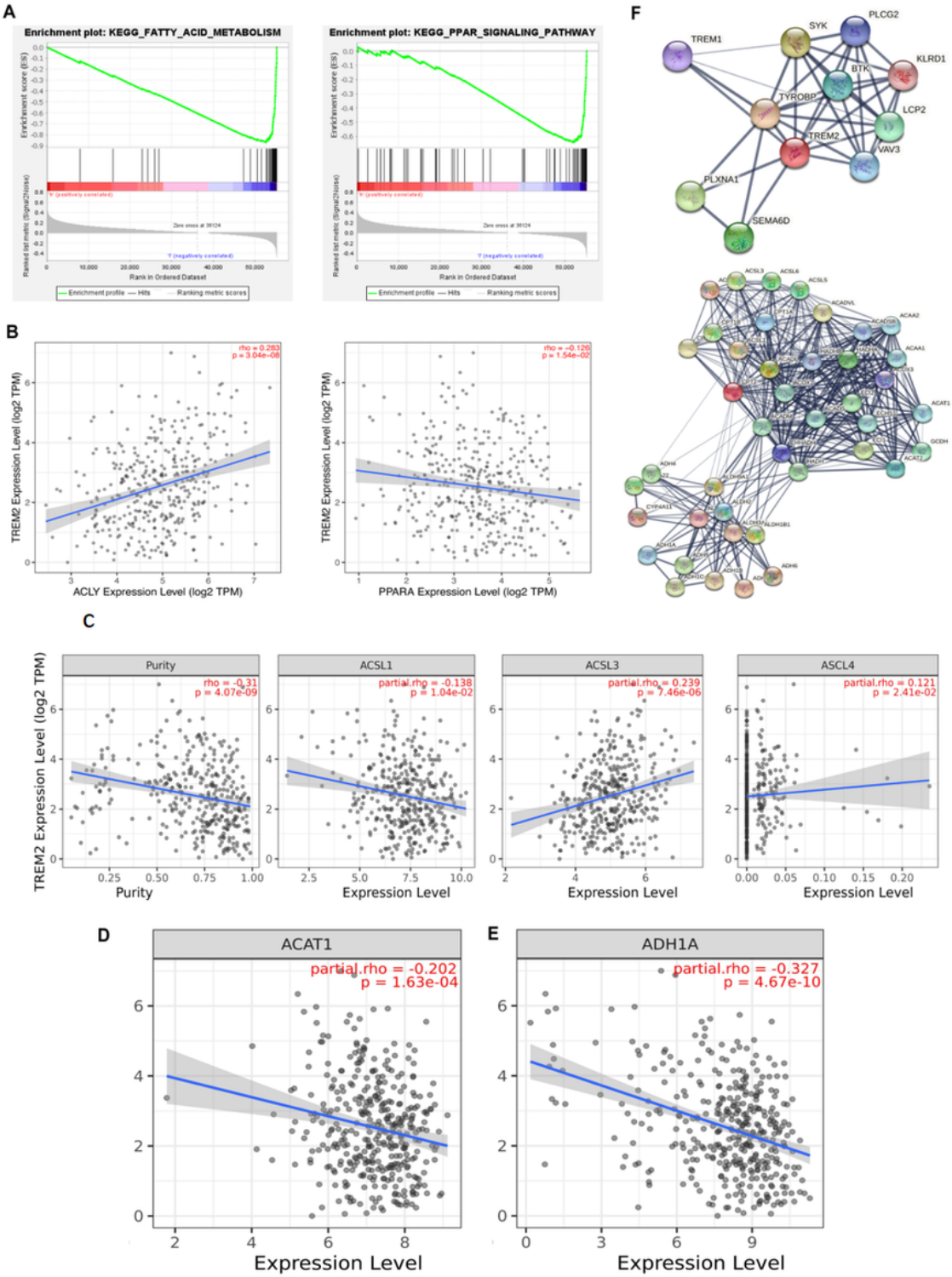

Figure 6

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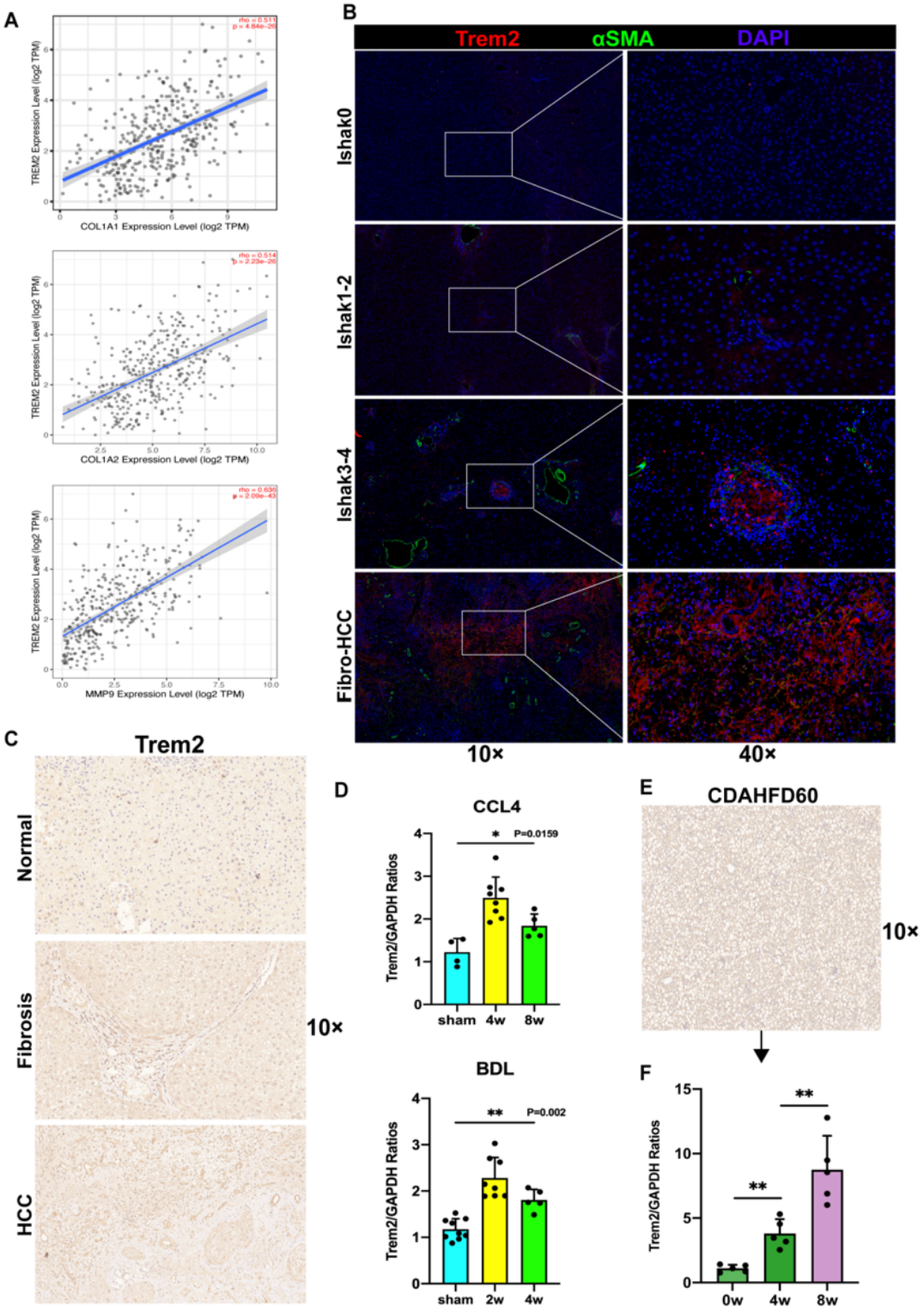

Figure 7

Caption not included with this version.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table2.xlsx