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Research Article

Upregulated HAVCR2: A Prognostic and Immune-Related Marker in Testicular Germ Cell Tumors

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Testicular germ cell tumors (TGCTs) are the most common solid malignant tumor in young men aged 20–34 years. Currently, the diagnosis and differentiation of TGCTs depend on immunohistochemical analysis, and the treatment methods are mainly surgery and chemoradiotherapy. Although immunotherapy has been applied in clinic, the response rate is not high, and also there is a lack of effective biomarkers. In this study, through high-throughput transcriptome sequencing and public data mining, the expression of hepatitis A virus cellular receptor 2 (HAVCR2) was found to be significantly upregulated in TGCT tissue and correlated with poor prognosis. Additionally, the expression level of HAVCR2 was negatively correlated with its DNA methylation but positively correlated with its copy number level. The HAVCR2 expression level was significantly positively correlated with the infiltration of six types of immune cells, including B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and myeloid dendritic cells. Also, the higher its expression level, the higher the immune cell abundance. Tumor immune dysfunction and exclusion (TIDE) scoring analysis showed that HAVCR2 was related to the responsiveness to TGCT immunotherapy. Drug sensitivity analysis revealed that HAVCR2 was related to the sensitivity of multiple antitumor drugs. This study demonstrated that HAVCR2 has high potential as a biomarker for the diagnosis, prognosis, and treatment of TGCTs.

1. Introduction

Testicular germ cell tumors (TGCTs), also known as type II germ cell tumors [1], are a common malignant tumor in urology, accounting for 95% of testicular malignant tumors, with the highest incidence among solid tumors in young men aged 18–35 years [2]. Since 1950, the incidence in many countries around the world has continued to increase, with the number of new cases more than doubling each year [3], also including the United States [4]. TGCTs are divided into two main types according to their histological characteristics: seminoma and nonseminoma [5]. At present, the diagnosis and differentiation of TGCTs depend on immunohistochemical analysis [6], and the treatment methods mainly include surgery, radiotherapy, and chemotherapy. The therapeutic effect is closely related to the tumor staging. The curative ratio in the first stage is 99%, while in the late stage, the curative ratio in patients with advanced disease and poor prognosis decreases from 90% to 50% [7]. Despite numerous successful cases in TGCT therapy over the past few decades, 10%–20% of patients still have incomplete responses or tumor recurrence [8], and 20%–30% exhibit the resistance to standard chemotherapy [9]. For cisplatin-refractory patients with such type of TGCTs, researchers have tried

different immunotherapy methods, among which PD-L1 and CTLA-4 immune checkpoint inhibitors are relatively common [10–12], but they fail to achieve good clinical results in refractory TGCTs [13], and only a few cases show good treatment outcomes [14]. Additionally, TGCT immunotherapy still lacks good markers [12]. Therefore, if a marker specific to TGCT immunotherapeutic response can be found, it will be of great significance to improve the treatment outcome of patients with refractory TGCTs.

Hepatitis A virus cellular receptor 2 (HAVCR2) encodes T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3). Its TIM gene family is located on 11B1.1 of the mouse genome and 5q33.2 of the human genome [15]. TIM-3 is a transmembrane receptor first discovered by Monney et al. [16] on the surface of interferon-γ (IFN-γ)-producing CD4+ T helper-1 (Th1) and CD8+ T cytotoxic-1 (Tc1) cells. In later studies, TIM-3 was found to be expressed in a variety of cells, including T lymphocytes, innate immune cells such as monocytes, natural killer (NK) cells and dendritic cells (DC), and cancer stem cells [17]. Subsequently, researchers also proved that TIM-3 can participate in tumor tolerance as an activation-induced inhibitory molecule [18, 19], and also induce T-cell exhaustion in slow virus infection and cancer [20]. Therefore, in the immunotherapy of solid tumors, TIM-3 is taken as a key target, and the research on the combined blockade of it with the PD-1 pathway is gradually deepening [21, 22]. Also, its function as a potential tumor prognostic biomarker in prostate cancer, renal clear cell carcinoma, colon cancer, bladder urothelial carcinoma, cervical cancer, and gastric cancer has also attracted the attention of researchers [15]. However, there has been yet no relevant research on the role and mechanism of HAVCR2 in TGCTs so far.

In this study, high-throughput transcriptome sequencing and bioinformatics analysis were adopted to determine that HAVCR2 is significantly upregulated in TGCT tissue. Then, the possible regulatory mechanism of HAVCR2 expression at multiple levels was analyzed. Finally, the analysis of the predictive value of HAVCR2 for the response to TGCT treatment was performed. This study provided a new idea and theoretical basis for the development of TGCT diagnostic, prognostic, and therapeutic markers.

2. Materials and Methods

2.1. Testicular Tumors and Paracancerous Tissue Samples. In this study, the specimens from seven cases of paracancerous tissue (as normal controls) and 15 cases of testicular germ cell tumor tissue used in this study were obtained from the Department of Urology, Hunan Cancer Hospital, the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University. The 15 patients ranged in age from 17 to 53 years, and the testicular histological types included three cases of nonseminoma and 12 cases of seminoma. All the tissue samples came from fresh frozen tumors. All specimens were examined by the Department of Histopathology in this hospital, and histopathological types were confirmed. All patients signed the written informed consent for tissue sample collection authorized by the ethics committee of Hunan Cancer Hospital.

2.2. RNA Extraction and Real-Time Fluorescent Quantitative PCR (qRT-PCR). Cell and tissue RNA were extracted by the Trizol method. One microliter of total RNA was taken, followed by the measurement of the OD value and RNA concentration by the NanoDrop 1000 Spectrophotometer. After the preparation of 1% agarose gel, 5 μl total RNA was taken for the determination of its integrity by electrophoresis. HAVCR2 expression was detected by the real-time quantitative polymerase chain reaction system (LightCyker 480, Roche, USA), and also the expression value of the internal reference gene (β-actin) was used as a standard reference. The reaction conditions were as follows: predenatured at 95°C for 5 min, followed by denaturation at 95°C for 10 s and annealing at 60°C for 10 s, and finally extended at 72°C for 10 s, amplified for 45 cycles, analyzed for CT value (threshold cycle value) by LightCyker 480 software and $2^{-\Delta\Delta}$ CT method, and calculated for HAVCR2 relative expression levels. The forward primer of HAVCR2 was TGTGCCTAACAGAGGTGTCC, and its reverse primer was CGACCTCCGCTCTGTATTCC; the forward primer of β-ACTIN was GGGAAATCGTGCGTGACATT, and its reverse primer was GGAACCGCTCATTGCCAAT.

2.3. Transcriptome Sequencing. For detailed materials and methods, please refer to a study published by our team in the early stage [23].

2.4. Bioinformatics Analysis. The Cancer Genome Atlas (TCGA) TGCT cohort dataset was downloaded from the Gene Expression Profiling Interactive Analysis (GEPIA2) database [24] and the University of California Santa Cruz (UCSC) Xena database [25]. GSE3218 dataset was downloaded from Gene Expression Omnibus (GEO) database. The differential methylation data of HAVCR2 were downloaded from the DiseaseMeth database [26]. DNA methylation and copy number variation of HAVCR2, as well as their corresponding expression data, were analyzed online and downloaded in the UCSC Xena database. The data related to DNA copy number variation and patient prognosis were also downloaded from the UCSC Xena database. The acquisition of gene data related to HAVCR2 expression and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed based on TCGA TGCT dataset analysis by the LinkedOmics online tool [27]. In order to analyze the correlation of HAVCR2 with the expression levels of immune cells and immune checkpoint molecules in TGCT tissue, a correlation analysis was conducted based on TCGA TGCT cohort data by using Assistant for Clinical Bioinformatics (https://www.aclbi.com/static/index.html#/). In order to obtain the evaluation of HAVCR2 for predicting the response to TGCT immune checkpoint therapy, TCGA TGCT cohort data were analyzed according to the tumor immune dysfunction and exclusion (TIDE) algorithm to derive the responsiveness of individual samples to immune checkpoint inhibitors [28]. The results of the above immune-related analysis were all processed by Hiplot online tool (https://hiplot.com.cn/) for data visualization.

Gene set cancer analysis (GSCA) was used to conduct an unbiased screening of the drugs potentially related to HAVCR2 [29]. This online tool integrates over 10,000 genomic data from TCGA across 33 cancer types and over 750 small molecule drugs from the Genomics of Drug Sensibility in Cancer (GDSC) and Cancer Therapeutics Response Portal (CTRP).

Based on TCGA TGCT cohort data, the online tool Assistant for Clinical Bioinformatics (https://www.aclbi.com/static/index.html#/) was adopted to analyze the correlation of HAVCR2 with expression levels of immune cells and immune checkpoint molecules in TGCT tissue. The analysis of HAVCR2 expression for predicting the response to immune checkpoint therapy for TGCT was also completed via the Assistant for Clinical Bioinformatics based on TCGA TGCT cohort data by using the TIDE algorithm analysis [28], and the data visualization was processed by the Hiplot online tool (https://hiplot.com.cn/) [30].

The Assistant for Clinical Bioinformatics online tool was also used to divide the samples into HAVCR2 high and low expression groups based on the TCGA TGCT cohort data with the median of HAVCR2 as the critical value. Then, the relevant functions of this tool were utilized to plot the mutation spectrum of the top 20 high-frequency mutant genes in these two groups of samples. Meanwhile, the mutation frequencies of three genes (KIT, KRAS, and PIK3CA) in the two groups of samples were compared. Finally, the analysis of the relationship between HAVCR2 expression and the common chemotherapy drugs paclitaxel and gemcitabine IC50 for TGCT was performed by this tool. Additionally, an unbiased screening for drugs potentially related to HAVCR2 was also performed by GSCA [29]. This online tool was used to integrate over 10,000 genome data from 33 cancer types from TCGA and over 750 small-molecule drugs from GDSC and CTRP. The data visualization was also completed by this online tool.

2.5. Data Analysis. All data were statistically analyzed by GraphPad Prism 5, and the Student's *t*-test was adopted to calculate the significance of differences between the two groups. One-way analysis of variance (ANOVA) and log-rank test were adopted to calculate the significant differences between multiple groups of data, as well as the overall survival time and the survival time of disease patients, respectively.

3. Results

3.1. The Significant Upregulation of HAVCR2 Expression Levels in TGCT Tissue. First, the expression of two transcripts of HAVCR2 genes (ENST00000522593 and TCONS_00165169) in TGCT patients and normal tissue was analyzed based on the transcriptome sequencing data published earlier by our team. HAVCR2 was significantly upregulated in TGCT tissue compared with normal tissue (Figures 1(a) and 1(b)). Then, the data on HAVCR2 expression in both TGCT and normal tissue were obtained from TCGA and Genotype-Tissue Expression (GTEx) databases. Also, it was found that HAVCR2 had a higher expression level in TGCT tissue (Figure 1(c)), and that there were both statistical differences in the expression between seminoma and nonseminoma and normal tissue (Figure 1(d)), which was consistent with the transcriptome sequencing data. In a set of TGCT gene expression profiling microarray data in GEO database (GSE3218), HAVCR2 expression in TGCT tissue was also significantly higher than that in normal tissue (Figure 1(e)). Finally, the above findings were verified in TGCT tissue samples by the qRT-PCR technique (Figure 1(f)).

3.2. The Better Diagnostic Efficacy and Prognostic Value that HAVCR2 Has for TGCT. In view of the significant difference in HAVCR2 expression between TGCT and normal testicular tissue, it was speculated that it may have the value as a diagnostic marker. Therefore, the receiver operating characteristic (ROC) curve analysis based on GSE3218 and qRT-PCR experimental data was performed, and the area under the curve (AUC) values of both curves were found to exceed 0.9, indicating that HAVCR2 has good diagnostic efficiency for TGCTs (Figures 2(a) and 2(b)). In the time dimension, the survival curve analysis revealed that the overall survival rate of patients with low HAVCR2 expression was higher than that of patients with high expression (Figure 2(c)). The ROC curve analysis showed that the HAVCR2 expression level has a good diagnostic value for judging the 5-year overall survival rate of TGCT patients (Figure 2(d)).

3.3. The Close Correlation of DNA Methylation and Copy Number with HAVCR2 Gene Expression. The expression of LncRNAs can be regulated by multiple mechanisms. In this study, the analysis at the epigenetic level revealed that the average methylation level of the HAVCR2 promoter region in normal tissue was significantly higher compared with TGCT tissue (Figure 3(a)). Correlation analysis revealed that the average DNA methylation level in the HAVCR2 promoter region was negatively correlated with HAVCR2 gene expression level, indicating that DNA methylation may be one of the important pathways for regulating gene expression (Figures 3(b) and 3(c)). Besides, it was also found that HAVCR2 copy number level was positively correlated with HAVCR2 gene expression level (Figures 3(d) and 3(e)). The survival curve showed that patients with the high copy number of HAVCR2 had a poor prognosis (Figure 3(f)), which may be due to the high HAVCR2 expression level in TGCT patients with the high copy number.

3.4. The Possibility that HAVCR2 in TGCT Is Involved in the Regulation of Multiple Signal Pathways. In order to determine the possible mechanism of HAVCR2 in TGCTs, Spearman's correlation analysis was adopted to screen the relevant genes (Figure 4(a)), and 50 genes with the most significant positive and negative correlations were exhibited in the heat map, respectively (Figure 4(b)). Through GO enrichment analysis, the following findings were obtained: in biological process, HAVCR2-related genes were mainly involved in biological regulation, metabolic process, multicellular biological process, localization, etc.; in cellular components, they mainly played a role in biofilm, nucleus, membrane-enclosed lumen, protein-binding complex, cytoplasm, etc.; in molecular functions, they were mainly involved in these processes such as protein binding, ion binding, nucleic acid binding, hydrolase activation, and transferase activation (Figure 4(c)). GSEA GO enrichment

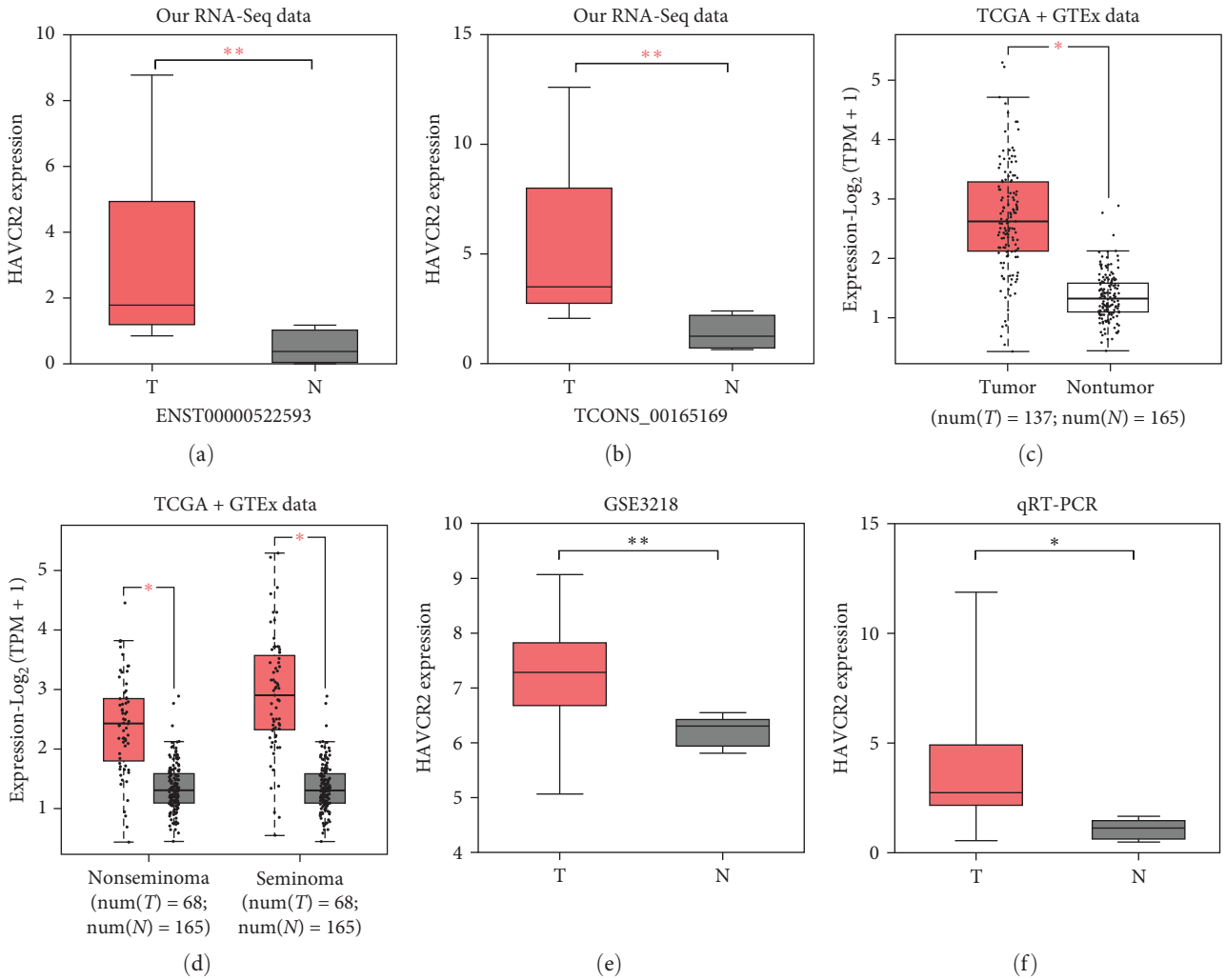


Figure 1: Upregulation of hepatitis A virus cellular receptor 2 (HAVCR2) in testicular germ cell tumors (TGCTs). (a/b) TGCT RNA sequencing data analysis revealed significant upregulation of two transcripts of HAVCR2, ENST00000522593 and TCONS_00165169, in the tumor group; gray represents normal testicular tissue and red represents tumor tissue. (c) The analysis of HAVCR2 expression level in TCGA TGCTs by using the GEPIA2 database, showing that HAVCR2 had a higher expression level in TGCT tissue. (d) The comparison of HAVCR2 expression levels between different TGCT subtypes and corresponding normal testicular tissue; gray represents normal testicular tissue and red represents nonseminoma (left) and seminoma (right); there were both significant differences between seminoma and nonseminoma and normal tissue. (e) The comparison of HAVCR2 expression levels between TGCT and corresponding normal tissue in the GSE3218 dataset, showing that HAVCR2 expression in TGCT tissue was also significantly higher than that in normal tissue. (f) The detection of HAVCR2 expression in TGCT tissue samples by qRT-PCR.

analysis was further performed and HAVCR2 was found to be related to the processes such as adaptive immune response, immune response regulation signal pathway, immune effect process regulation, T-cell activation, bone morphogenetic protein stimulation pathway, the establishment of tissue polarity, and spinal cord development (Figure 4(d)). GSEA KEGG enrichment analysis revealed that these genes were mainly related to the signal pathways such as tuberculosis, cell adhesion molecules, phagosomes, EB virus infection, NOD-like receptor signal, T-cell receptor signal, Th cell differentiation, hippo signal receptor, and cell cycle (Figure 4(e)).

3.5. The Close Correlation of HAVCR2 Gene Expression with Tumor Immunity. Given that HAVCR2 was found to be related to multiple immune signal pathways in the aforementioned enrichment analysis, it was analyzed which immune cells and molecules HAVCR2 is specifically related to. The data showed that the HAVCR2 expression level was closely related to six types of immune cells (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and myeloid dendritic cells), and that the higher the HAVCR2 expression level, the higher the immune cell abundance (Figure 5(a)). In the samples with high HAVCR2 expression, the infiltration degree of the above six immune cells was higher (Figure 5(b)). Furthermore, eight important immune checkpoint molecules were also selected to observe their correlation with HAVCR2 expression. It was found that in the samples with high HAVCR2 expression, the expression level of immune checkpoint molecules was also higher (Figure 5(c)).

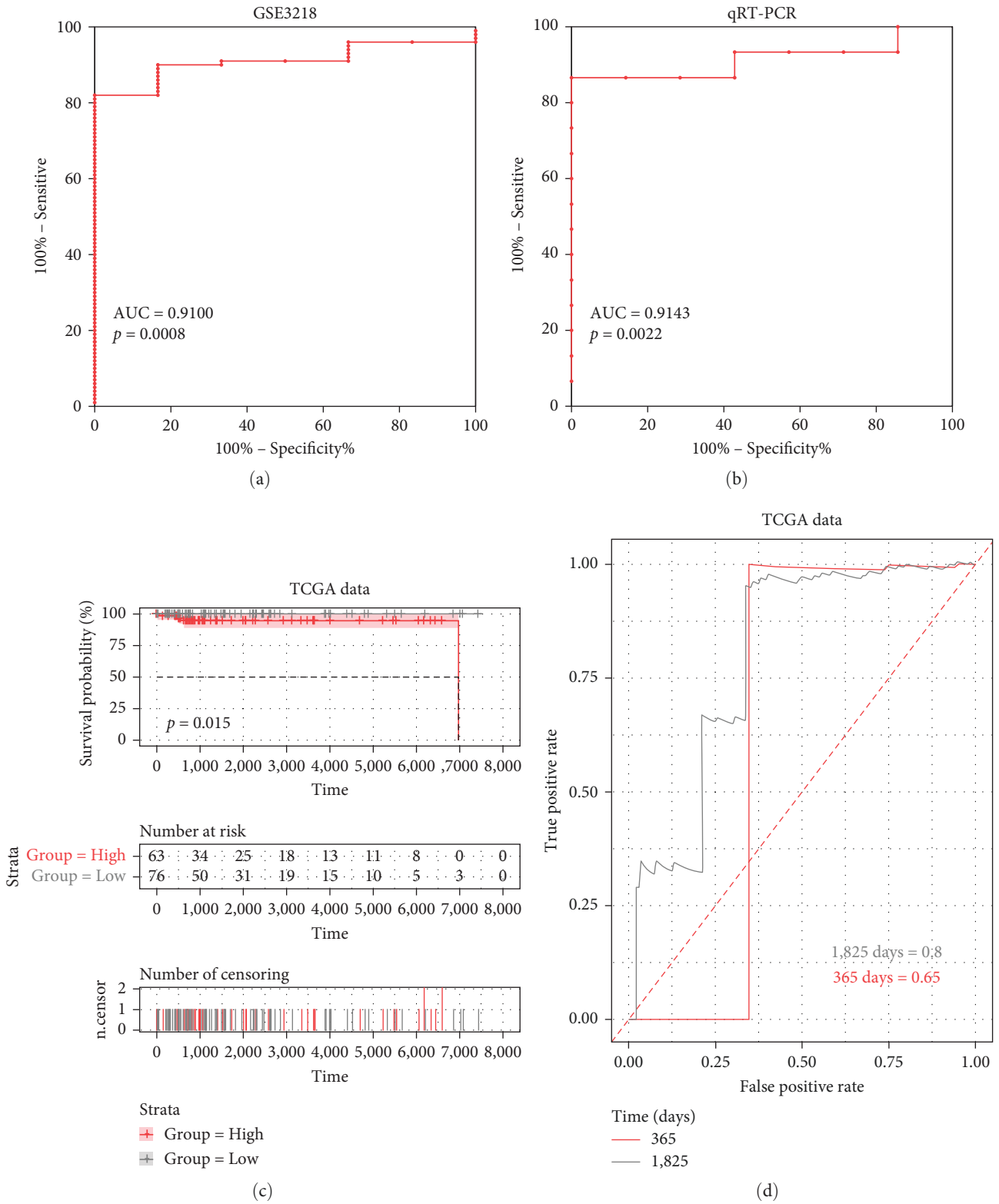


Figure 2: Hepatitis A virus cellular receptor 2 (HAVCR2) as a potential diagnostic and prognostic marker of testicular germ cell tumors (TGCTs). (a/b) The receiver operating characteristic (ROC) curves based on HAVCR2 expression were plotted according to GSE3218 and qRT-PCR data, respectively, and the AUC values of both curves exceeded 0.9. (c) The data of the TCGA TGCT cohort were used to divide clinical samples into two groups of high and low expression based on HAVCR2 expression for survival analysis, showing that the overall survival rate of patients with low HAVCR2 expression was higher than that of those with high expression. Gray represents HAVCR2 low expression group and red represents HAVCR2 high expression group. (d) The receiver operating characteristic (ROC) curve of HAVCR2 for judging the prognosis of TGCT patients, showing that the expression level of HAVCR2 had a good diagnostic value for judging the 5-year overall survival rate of TGCT patients.

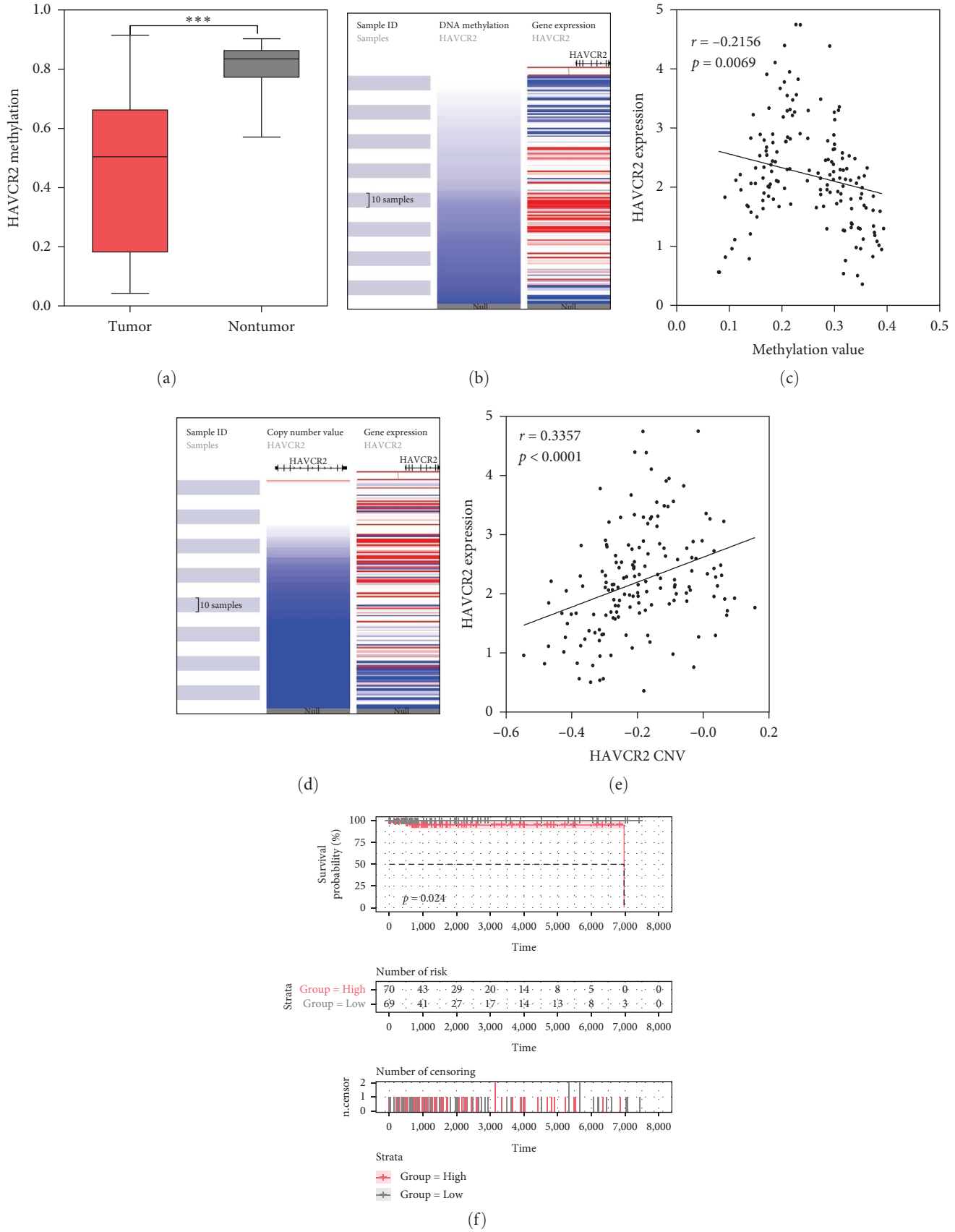


Figure 3: The methylation and copy number variation of hepatitis A virus cellular receptor 2 (HAVCR2). (a) The analysis of the methylation level of HAVCR2 in normal tissue and testicular germ cell tumor (TGCT) patients by using the DiseaseMeth database, showing that the average methylation level of the HAVCR2 promoter region in normal tissue was significantly higher compared with TGCT tissue. (b) The

plotting of both the expression and promoter region methylation profiles of HAVCR2 by using UCSC Xena online tool. (c) Correlation analysis between HAVCR2 mRNA expression and average methylation in the HAVCR2 promoter region. (d) The plotting of the heat map of the expression and copy number of HAVCR2 by using UCSC Xena online tool. (e) Correlation analysis between HAVCR2 mRNA expression and its DNA copy number variation. (f) Based on TCGA TGCT cohort data, clinical samples were divided into two groups of high and low DNA copy number for survival analysis. The survival curve showed that patients with the high copy number of HAVCR2 had a relatively poor prognosis.

3.6. The Potential of HAVCR2 as a Therapeutic Marker for TGCT Immune Checkpoint. Since HAVCR2 is related to a variety of immune cells and immune checkpoint molecules in TGCTs, the possibility of HAVCR2 as a therapeutic marker for immune checkpoint molecules in TGCTs was further explored. The TIDE algorithm was used for analysis, and the TIDE value corresponding to high HAVCR2 expression was found to be lower than that of the low expression group (Figure 6(a)). Meanwhile, the patients with high HAVCR2 expression are more likely to benefit from immune checkpoint therapy (Figure 6(b)), and also based on HAVCR2 expression, the benefit and nonbenefit populations of immune checkpoint therapy can be well differentiated (Figure 6(c)). Besides, the HAVCR2 expression level was positively correlated with the content of effector T-cell-related factor IFN-γ (Figure 6(d)), and also HAVCR2 expression was higher in samples with cytotoxic T lymphocytes flag (Figure 6(e)). It was also found that the HAVCR2 expression was significantly positively correlated with immune dysfunction scoring (Figure 6(f)). All the evidence pointed to the potential of HAVCR2 as a marker for TGCT immune checkpoint therapy.

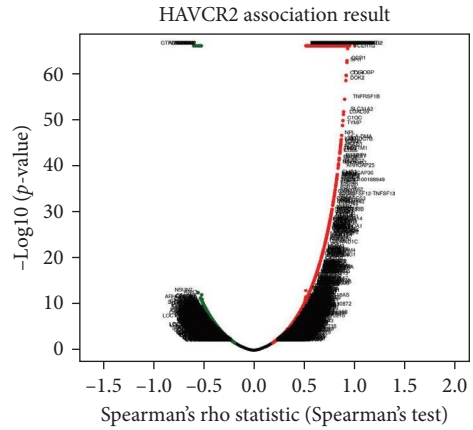
3.7. The Correlation of the Mutation Spectrum of HAVCR2-Related Genomes and Their Expression with the Sensitivity of Common Chemotherapy Drugs. Based on the median of HAVCR2 expression in TCGA TGCT cohort data, these TGCT tissue samples were divided into HAVCR2 high and low expression groups, with the genome mutation spectrum of the two groups of samples plotted. It was found that the mutation frequency of kitproto-oncogeneprotein (KIT) (belonging to type 3 receptor tyrosine kinase) in samples of the HAVCR2 high expression group was as high as 23% and that of Kirsten rat sarcoma viral oncogene homolog (KRAS) was 17%, while that of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) was only 5% (Figure 7(a)). The KIT mutation frequency in samples of the HAVCR2 low expression group was 8% (Figure 7(b)). There were significant differences in the mutation status of KIT, KRAS, and PIK3CA between samples of HAVCR2 high and low expression groups (Figure 7(c)). Different gene mutations can lead to different sensitivity of patients to drugs. In this study, follow-up analysis was performed to find that there were significant differences in the sensitivity of samples in HAVCR2 high and low expression groups to the chemotherapy drugs paclitaxel (microtubule inhibitor in mitosis) and gemcitabine (pyrimidine antitumor drugs) (Figure 7(d)), with the negative correlation of gemcitabine IC50 with HAVCR2 expression (Figure 7(e)). These results suggested that HAVCR2 may be an important indicator of the sensitivity of mutation-targeting drugs and common chemotherapy ones.

4. Discussion

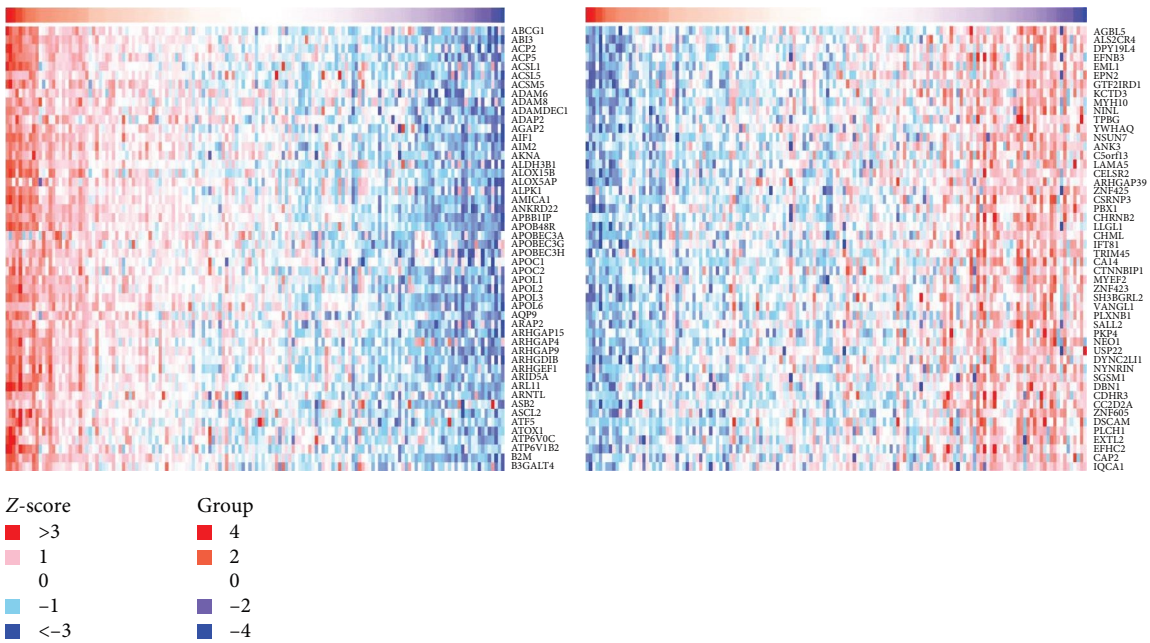
This study showed that HAVCR2 expression levels were significantly upregulated in TGCT tissue, compared with adjacent cancer tissue, which was confirmed by all the analysis results in different databases. It was further found that HAVCR2 has better diagnostic efficacy and prognostic value for TGCTs, and that the patients with low HAVCR2 expression have a better prognosis, suggesting that HAVCR2 can be used as a potential prognostic biomarker for TGCTs. A growing number of studies have shown that molecules associated with cancer carcinogenesis and metastasis can be used not only as potential prognostic markers but also as therapeutic targets [31]. Similarly, many studies have proved that HAVCR2 is significantly upregulated in different tumor tissues, and also closely related to the prognosis of urothelial carcinoma of bladder [32], gastric carcinoma [33], renal clear cell carcinoma [34], pancreatic cancer [35], and cervical cancer [36], suggesting that HAVCR2 may play an important role in different types of cancer.

In order to further explore the regulatory mechanism of HAVCR2, relevant analysis was conducted. It was found that the HAVCR2 gene expression level was negatively correlated with DNA methylation but positively correlated with its copy number. In melanoma, Holderried et al. [37] detected a significant negative correlation between the mRNA expression and methylation in the HAVCR2 promoter region, confirming that DNA methylation can regulate the HAVCR2 gene at the epigenetic level. In terms of the copy number, studies showed that the DNA copy number and mRNA expression of HAVCR2 in head and neck cancer were significantly increased compared with the control group [38]. This suggested that the changes in the methylation and copy number of HAVCR2 may be one of the important reasons for the upregulation of HAVCR2 expression.

HAVCR2 encodes TIM-3 protein, and the latter has gradually become an important immune checkpoint molecule, by which immunotherapeutic drugs for a variety of malignant tumors are being developed [39]. Through the analysis of various online databases, HAVCR2 was found to be involved in the regulation of multiple signal pathways, such as adaptive immune response, immune response regulatory signal pathway, and T-cell activation. So, is there a possibility of HAVCR2 as a marker for immune checkpoint therapy in TGCTs? Interestingly, based on the analysis of tissue samples from TGCT patients, HAVCR2 gene expression was found to be closely related to tumor immune cells. The higher the HAVCR2 expression level, the higher the abundance of B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and myeloid dendritic cells. Meanwhile, it was also found that eight immune checkpoint molecules



(a)



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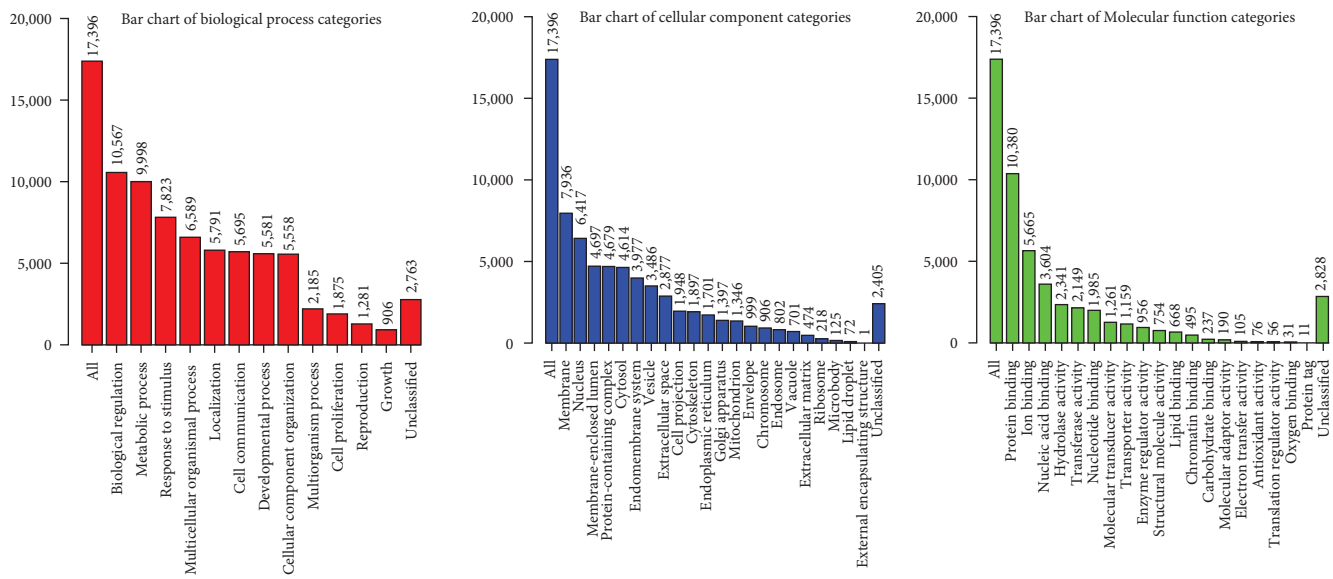


(c)

Figure 4: Continued.

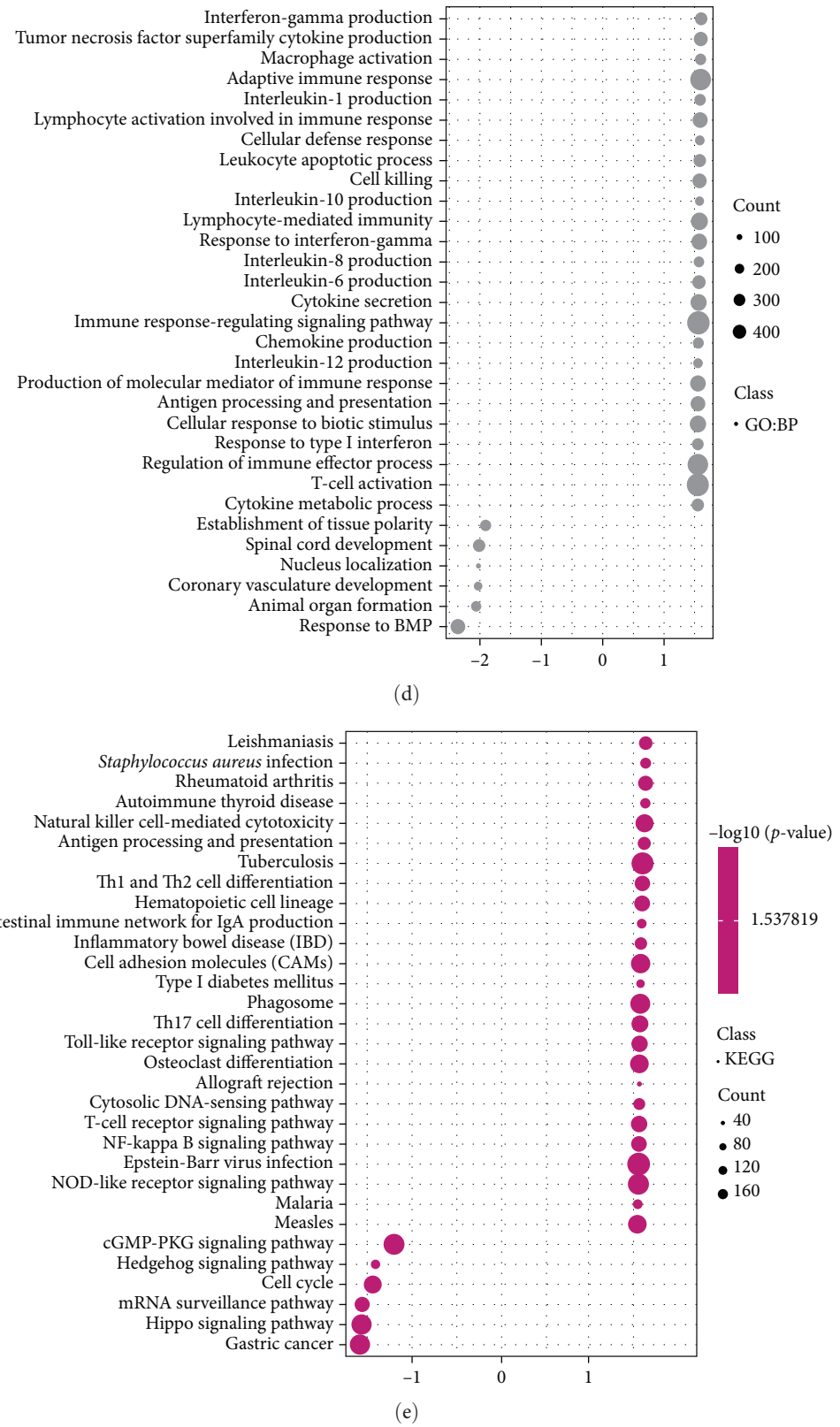


Figure 4: Coexpressed genes and their related signal pathways of hepatitis A virus cellular receptor 2 (HAVCR2). (a) The exhibition of the genes related to HAVCR2 expression in TCGA testicular germ cell tumor (TGCT) data by Volcano map. (b) The heat map of the top 50 genes most significantly positively and negatively correlated with HAVCR2. (c) The exhibition of the GO term enriched by the coexpressed genes of HAVCR2 by bar plot. (d) GO-BP enrichment analysis of coexpressed genes of HAVCR2. (e) KEGG enrichment analysis of HAVCR2 coexpressed gene.

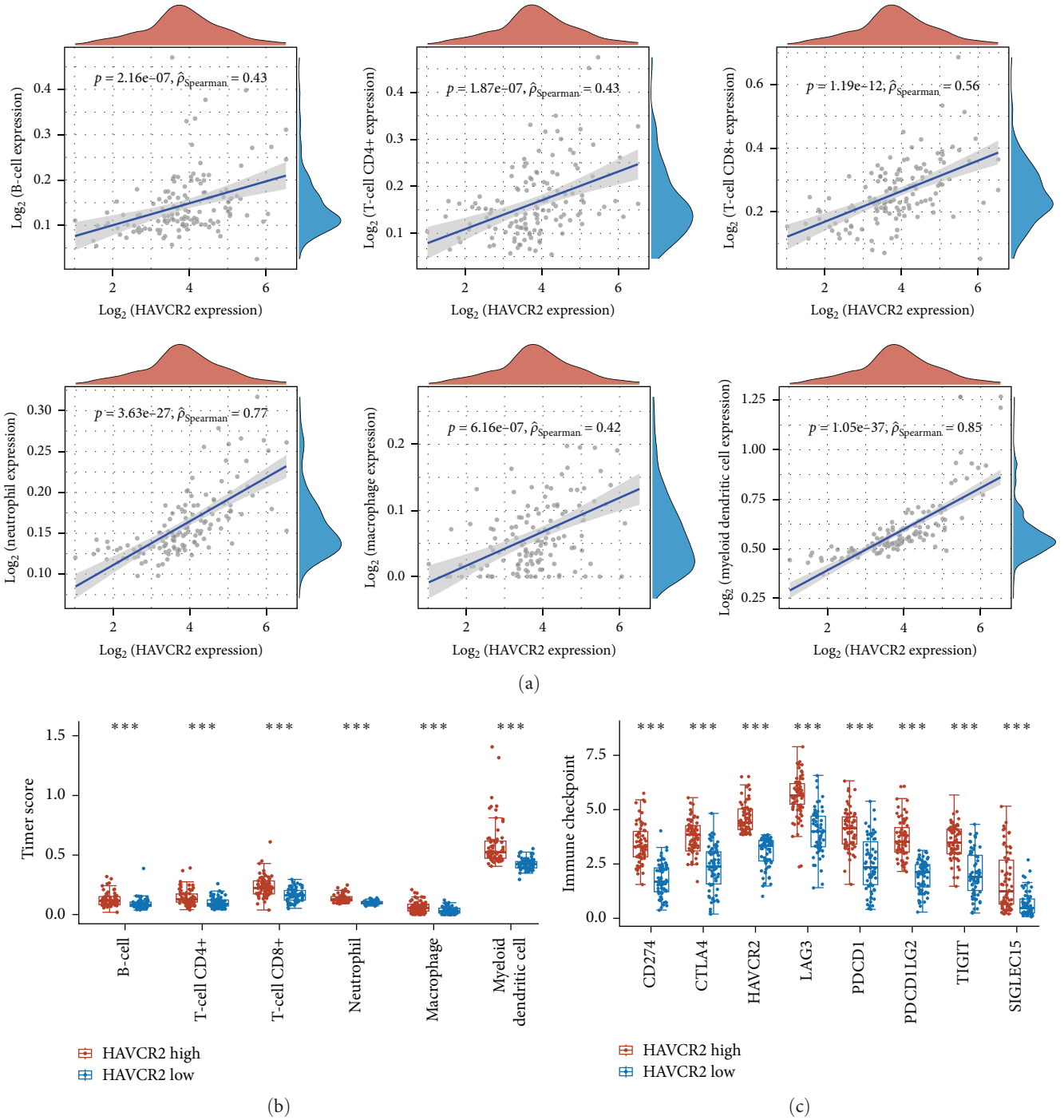


Figure 5: Correlation between hepatitis A virus cellular receptor 2 (HAVCR2) and immune cells and immune checkpoint molecules in testicular germ cell tumor (TGCT) tissue. (a) The significant negative correlation of HAVCR2 expression with the scoring of B cells, CD4+ T cells, CD8+T cells, macrophages, neutrophils, and dendritic cells. (b) Higher scores for B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils, and dendritic cells in the samples of high HAVCR2 expression group. (c) Higher expression levels of immune checkpoint molecules in testicular germ cell tumors (TGCT) patients with high HAVCR2 expression.

were positively correlated with HAVCR2 expression levels in TGCTs.

In the case of ineffective conventional chemotherapy, immunotherapy (e.g., PD-1 inhibitor) is regarded as an effective scheme for the treatment of solid tumors. At present, in a number of clinical studies and experiments, anti-PD-1 immunotherapy has been reported [5, 10–12]. Regrettably, immune checkpoint inhibition therapy (including PD-L1 and CTLA-4 inhibitors) has not shown an excellent therapeutic effect, and the effect that it exerts in the treatment of refractory germ cell tumors outside clinical trials is still limited [13]. An important reason for this is the lack of effective

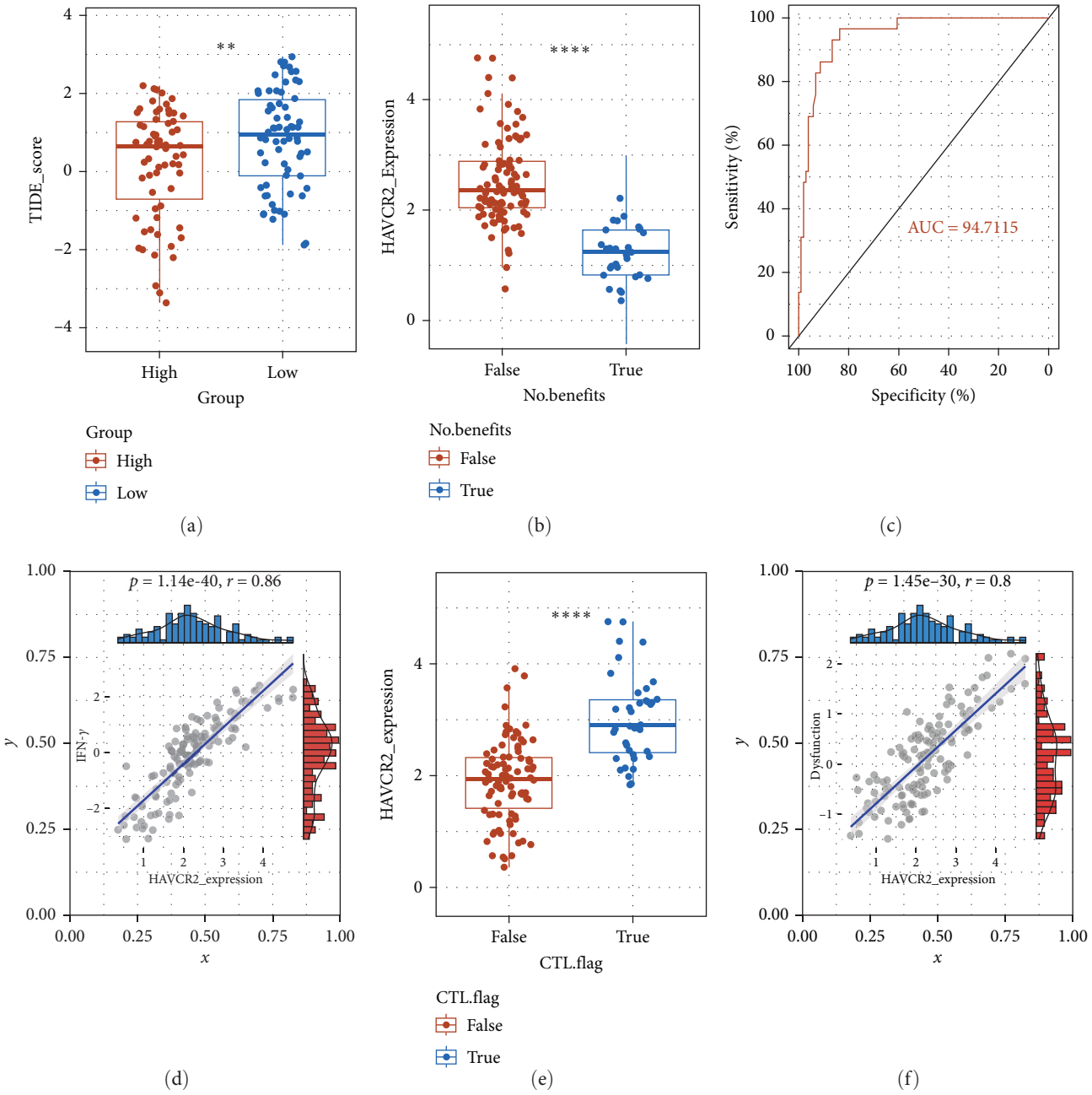
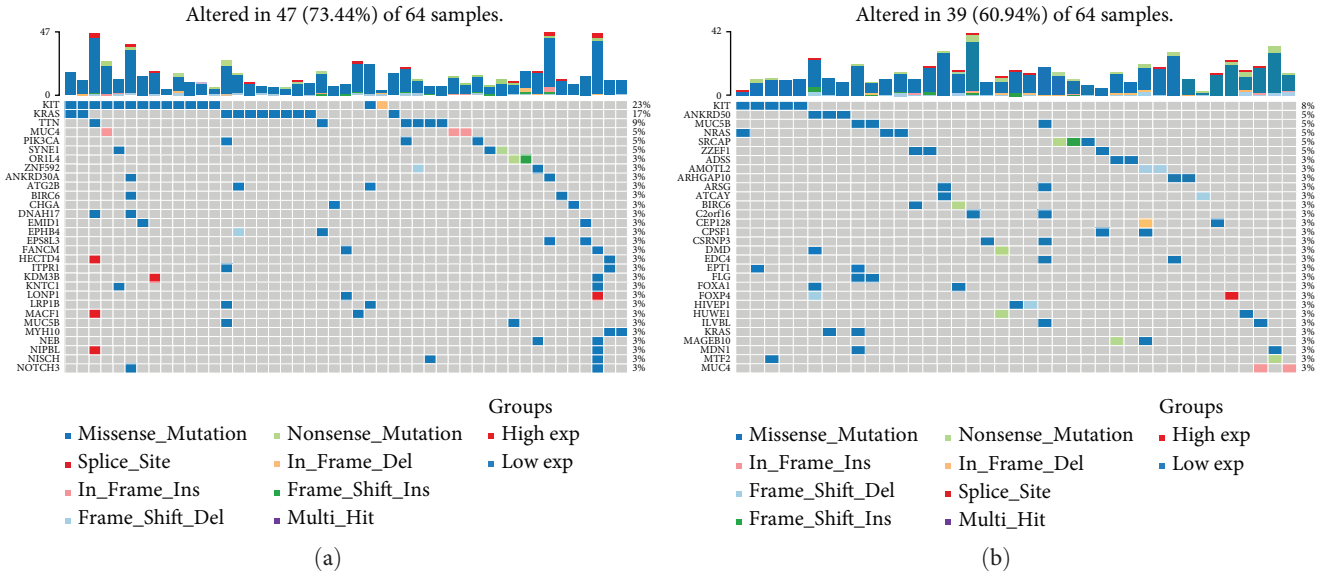
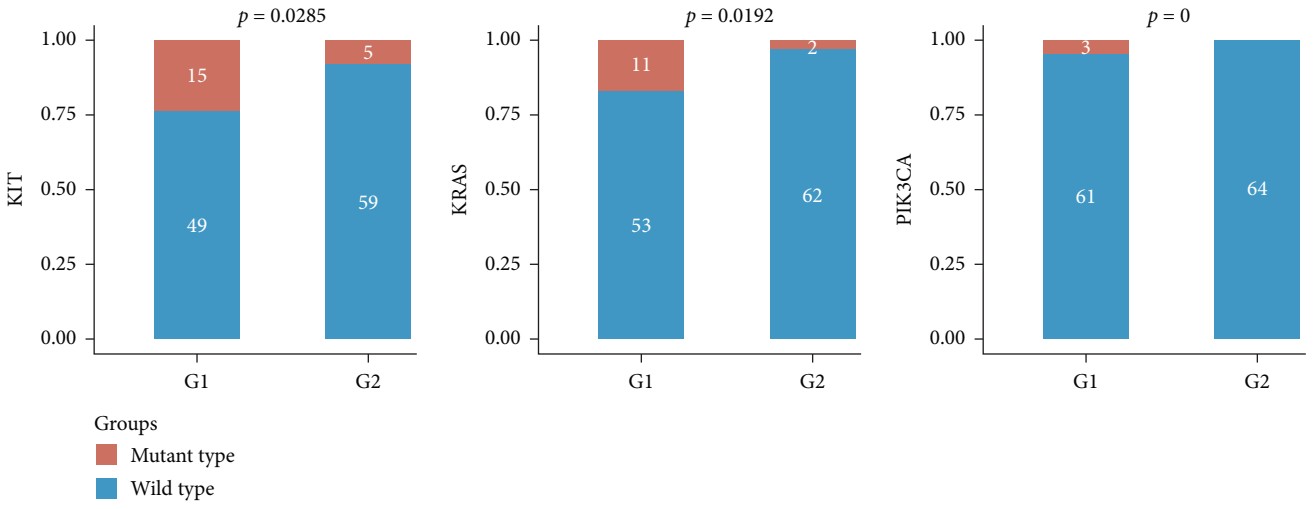


Figure 6: The assessment of the value of hepatitis A virus cellular receptor 2 (HAVCR2) for predicting the response to TGCT immune checkpoint therapy by using tumor immune dysfunction and exclusion (TIDE) algorithm. (a) Lower TIDE scores in patients with high HAVCR2 expression. (b) Higher levels of HAVCR2 expression in populations potentially benefiting from immune checkpoint therapy. (c) Receiver operating characteristic (ROC) curve analysis of the sensitivity and specificity of HAVCR2 expression in judging whether patients can benefit from immune checkpoint therapy. (d) The positive correlation between the HAVCR2 expression and IFN-γ expression. (e) The higher HAVCR2 expression in the samples with cytotoxic lymphocyte flag. (f) The positive correlation of the HAVCR2 expression level with immune dysfunction scores.

predictors for clinical immunotherapy decision making, and it is difficult for clinicians to determine under what circumstances TGCT patients can be treated with immunotherapy and which immunotherapeutic drugs are chosen to treat them. Therefore, it was further explored whether HAVCR2 can be used as a marker for immune checkpoint therapy in TGCTs. Through the analysis of the TIDE algorithm and ROC curve, it was further proved that HAVCR2 has good potential as a marker of immunotherapeutic response. Moreover, the reason for the failure of PD-1 immunotherapy may be that the inhibition of the PD-1 pathway is not sufficient to overcome the dysfunction of depleted T cells, resulting in the failure of PD-1 single drug immunotherapy to block TCGT. In this case, the combined blockade of PD-1 and TIM-3 may





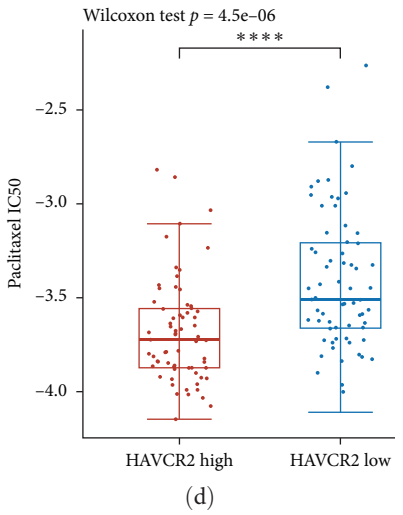
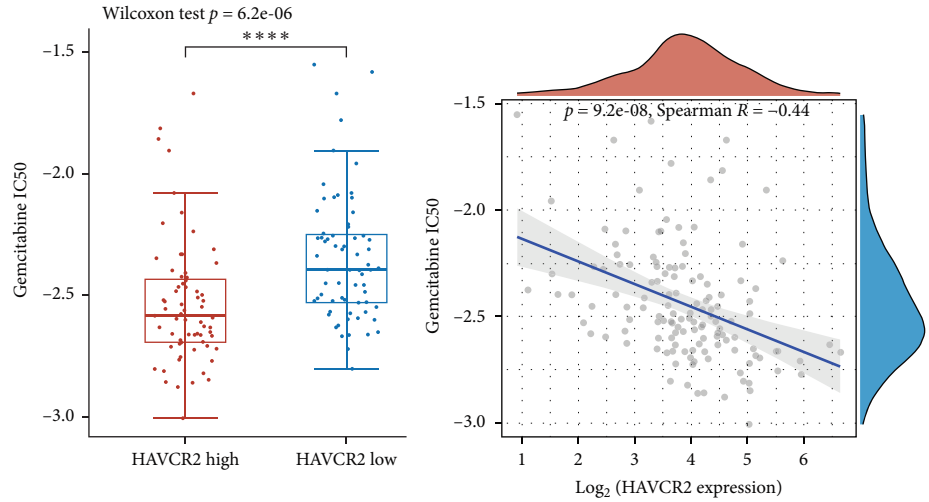


Figure 7: Continued.



(e)

Figure 7: The differences in genome mutation spectrum and sensitivity of common chemotherapy drugs between samples in hepatitis A virus cellular receptor 2 (HAVCR2) high and low expression groups. (a/b) The genome mutation status of patients in HAVCR2 high and low expression groups. The genes and samples were sorted by mutation frequency and disease histology, respectively; as shown in the annotation column (bottom), the left side indicates the mutant gene, each column represents a sample, and the right side indicates the mutation frequency of this gene in all samples; the mutation information for each gene in each sample is shown in the figure and different colors with specific notes at the bottom represent various mutation types. (c) The mutation status of kitproto-oncogeneprotein (KIT), Kirsten rat sarcoma viral oncogene homolog (KRAS), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) genes in samples of HAVCR2 high and low expression groups. The abscissa represents different groups of samples; the ordinate represents the proportion of mutant and wild-type samples; different colors represent different mutation types; the upper side represents the significance *P*-value. (d) The lower level of paclitaxel IC50 in the HAVCR2 high expression group. (e) The negative correlation of HAVCR2 expression levels with the drug sensitivity of gemcitabine (Note: G1 represents HAVCR2 high; G2 represents HAVCR2 low).

be one of the effective schemes for overcoming the resistance to anti-PD-1 therapy [15, 40, 41], which also provides a new idea for TGCT immunotherapy.

The mutant KIT causes changes in its expression pattern and internal kinase structure, which relieves the automatic inhibitory state, with ligand-independent autophosphorylation/independent of ligand autophosphorylation, and activates the downstream proliferation pathway; this results in unregulated cell proliferation, thus triggering tumorigenesis [42]. After the pathogenic mutation in KRAS genes occurs, they will always remain bound to GTP. Also, KRAS proteins will be in a state of ceaseless activation and continuously activate downstream signal pathways such as PI3K, RAF-MEK-ERK (MAPK), and Ral-GEF, which, in turn, stimulates cell proliferation and migration, thereby leading to tumorigenesis [43]. PIK3CA gene mutation can cause PI3K enzymes to remain in a state of continuous activation, enhance intracellular signal transduction, and incur a series of diseases, including cancer, autoimmune disorders, and hematopoietic system diseases. The activation mutation in PIK3CA was found in approximately 30%–40% of cancer patients [44]. Alan McIntyre demonstrated the relationship between the activation mutation, copy number, and expression levels of KRAS2, KIT, ERBB2, and GRB7, indicating their involvement in TGCT cellular behavior. Paclitaxel can promote microtubule polymerization and stabilize the drugs that have polymerized microtubules [45]. Therefore, KIT, KRAS, and PIK3CA may be relatively good targets for intervention treatment. HAVCR2 is possibly an important indicator of the sensitivity of multitarget inhibitors and common chemotherapy drugs.

About 1%–5% of TGCT patients are resistant to platinum chemotherapy. Patients with recurrent TGCTs usually receive high-dose chemotherapy, but this treatment can lead to serious adverse reactions and cytotoxicity. However, there are multiple epigenetic alterations in TGCTs, for which targeted therapy can be implemented through specific therapeutic methods. Several studies have explored the correlation between tumor progression and antineoplastic drug resistance, which may help improve chemotherapy for cancer species [46, 47]. Histone deacetylase (HDAC1) is identified as the main target for TGCT therapy [48]. Lobo et al. [49] found that HDAC inhibitor belinostat (50 nM to 1 μM) effectively reduced the dose- and time-dependent effects of cell viability (cisplatin sensitivity and resistant clones) over a period of time. Bromodomain and extraterminal domain (BET) inhibitors can restrict the gene expression of growth-promoting factors and oncogenic factors (e.g., MYC), thereby inducing cell cycle arrest and apoptosis in different cancer models. Jostes et al. [50] found in studies that BET inhibitors can induce apoptosis and G1 cell cycle arrest in platinum-resistant TGCT cell lines. Cell cycle-related kinases (CDK1/2/4/6) that directly regulate cell cycle progression have been extensively studied in preclinical and clinical trials of TGCTs and other types of cancer with different CDK inhibitors used. As a central regulator of proliferation signal transduction, mammalian target of rapamycin (mTOR) is an ideal target for tumor therapy. Schaffrath et al. [51] believed that the inhibition of

the PI3K/AKT/mTOR pathway may be a target for TGCT overcoming cisplatin resistance. Gutekunst et al. [52] demonstrated the close relationship between p53 protein levels and the degree of apoptosis in pluripotent TGCT cells, and also p53 proapoptotic function mediated by NOXA and PUMA. Therefore, HAVCR2 may be an important marker for drug sensitivity in TGCT patients. The detection of HAVCR2 expression level has a certain guiding significance for the selection of clinical chemotherapy drugs.

In this study, we demonstrated a significant upregulation of HAVCR2 expression in TGCT tissues. Based on its expression levels, HAVCR2 shows promise as a diagnostic marker for tumor types and is correlated with a poor prognosis. Additionally, HAVCR2 has the potential to serve as a prognostic marker. Our findings also revealed the association of HAVCR2 with tumor immunity and its impact on the sensitivity of TGCT cells to various antitumor drugs. These results suggest that HAVCR2 could be a valuable therapeutic target for immunotherapy and drug treatment in TGCT. This study provides novel. However, there are still some deficiencies in this study. Since TCGT is a relatively uncommon tumor, enough samples or public data were not available to adequately verify the findings from this study. Therefore, further verification is required for them in the future insights into tumor progression and antitumor drug resistance in TGCT, which may contribute to the improvement of TGCT treatment strategies.

Moving forward, our next steps involve expanding the sample size to validate the clinical relevance of HAVCR2. By collecting a sufficient number of samples, we aim to further support the application of HAVCR2 as a marker with enhanced clinical utility.

In conclusion, this study showed that the HAVCR2 expression level was significantly upregulated in TGCT tissue, and that based on its expression level, tumor types could be diagnosed very well, with quite good judgment on patient prognosis. Meanwhile, it also revealed that this molecule was related to TGCT tumor immunity and the sensitivity of various antitumor drugs. These data suggest that HAVCR2 may be a very good marker for diagnosis, prognosis, immunotherapy, and drug therapy of TGCTs.

Data Availability

Basic data supporting the results can be obtained from the corresponding author on reasonable request; email: 1172881652@qq.com.

Conflicts of Interest

The author declares that there are no potential conflicts of interest about this article.

Authors' Contributions

JG supervised the study. HB and LL conceived the study and designed the experiments. LX, YX, and YW performed data analysis and generated the figures and tables. XZ and HJ wrote the manuscript with input from HB, YL, ZL, and JC assisted with language revisions. All authors contributed to the article and approved the submitted version.

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