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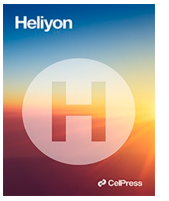

Comprehensive analysis of the expression of the IGF2BPs gene family in head and neck squamous cell carcinoma: Association with prognostic value and tumor immunity

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ABSTRACT

Background: Head and neck squamous cell carcinoma (HNSCC) represents a predominant type of cancer found in the head and neck region, characterized by a high incidence and unfavorable prognosis. The IGF2BPs gene family, which belongs to the RNA-binding protein class, has been critically implicated in several cancers, and its involvement in HNSCC necessitates further exploration.

Objective: To explore the clinical significance and potential biological functions of the IGF2BPs gene family in HNSCC.

Methods: A bioinformatic methodology was employed to examine the expression profile, diagnostic and prognostic significance, and biological mechanisms of the IGF2BPs gene family in HNSCC, with a particular emphasis on its involvement in the immune function of HNSCC. This was followed by in vitro investigations to unravel the biological roles of the IGF2BPs gene family in HNSCC.

Results: This investigation has demonstrated that, in contrast with normal control tissue, HNSCC has a substantial elevation in the expression level of the IGF2BPs gene family. Patients with a high level of IGF2BPs gene family expression demonstrated higher prediction accuracy for HNSCC. Furthermore, patients with HNSCC and elevated IGF2BPs gene family expression levels exhibited poor survival outcomes. The IGF2BPs gene family displayed a significant association with a variety of immune infiltrating cells and immune genes in HNSCC. Studies conducted in vitro have confirmed that IGF2BP2 silencing suppressed the migration, proliferation, and invasion of HNSCC cells.

Conclusions: It has been determined that the IGF2BPs gene family plays a crucial part in the onset and progression of HNSCC, and its association with tumor immunity has been established. The IGF2BPs gene family holds promising potential as a diagnostic and prognostic biomarker for HNSCC.

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1. Introduction

Head and neck cancer represents a group of malignant tumors that manifest in diverse anatomic areas of the upper gastrointestinal tract. Globally, over 830,000 new cases of head and neck cancer are reported to be diagnosed each year, with head and neck squamous cell carcinoma (HNSCC) accounting for more than 95 % of cases. The primary risk contributors to HNSCC are smoking, betel nut consumption, alcohol consumption, genetic susceptibility, and human papillomavirus (HPV) infection [1]. Despite significant progress in HNSCC research and therapy, the primary treatment for HNSCC patients still relies on surgical resection along with radiotherapy, chemotherapy, and immunotherapy [2]. The 5-year survival rate is still below 50 % because HNSCC is aggressive, metastatic, and recurring [3]. Gene targeting therapy has demonstrated promise in the treatment of HNSCC in recent years [4]. However, HNSCC development is a complicated process that involves various genes. Therefore, finding new targets is crucial for improving the care and prognosis of individuals with HNSCC.

Insulin-like growth factor 2 mRNA binding proteins (IGF2BPs), also known as IMPs, comprise three members: IGF2BP1, IGF2BP2, and IGF2BP3. They belong to the family of oncofetal RNA-binding proteins (RBPs), are encoded by the IGF2BPs gene family, and considerably conserved, and exert significant regulatory control over RNA processing, at various levels, such as localization, translation, and stability [5]. The IGF2BPs gene family is predominantly expressed during mammalian embryonic development, and its contributions to pivotal cellular processes, such as cell proliferation, differentiation, and metabolism, have been established [6]. The IGF2BPs gene family plays important roles in cancer by regulating mRNA stability and translation, promoting invasion and metastasis, modulating EMT, and potentially contributing to drug resistance. The scientific community has recently identified the IGF2BPs gene family as being dysregulated in several tumor tissues, including lung, liver, and colon cancers. The dysregulation of the IGF2BPs gene family further enhances the proliferation, migration, and invasive capabilities of tumor cells, thereby establishing a link between the IGF2BPs gene family and a poor prognosis [7–14]. Previous literature has also shown that the IGF2BPs gene family is dysregulated in HNSCC and plays an important role in the tumor metabolism and other processes of HNSCC [15]. However, despite the advancements in cancer research, a limited understanding of the potential mechanisms of the IGF2BPs gene family is available. Consequently, there is a pressing need to unravel its expression patterns and molecular functions in HNSCC.

The incorporation of bioinformatics in cancer research provides invaluable insights by analyzing complex genomic data, thereby enabling the identification of novel biomarkers, potential drug targets, and personalized treatment strategies for improved patient outcomes. This research used bioinformatics technologies to examine the relationship between three IGF2BPs gene family members

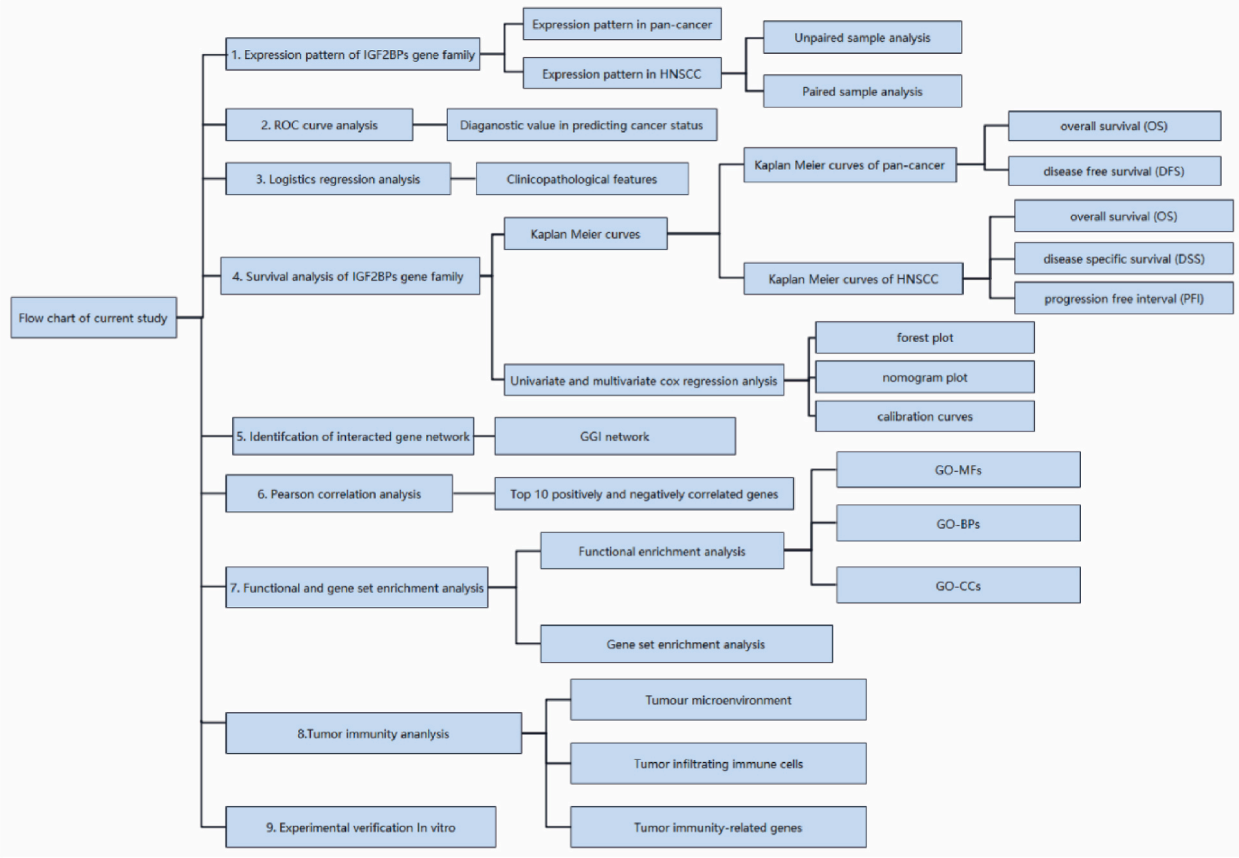

Fig. 1. The current study design was illustrated using a schematic diagram.

and HNSCC. Furthermore, the biological significance of IGF2BP2 in HNSCC was confirmed through in vitro experiments. The present investigation utilized information from the TCGA database, HPA database, and GEPIA2 database to explore the association of 3 genes in the IGF2BPs gene family with expression pattern, clinical characteristics, prognostic value, diagnostic value, associated genes, biofunctional enrichment, and tumor immunity in HNSCC patients. To further examine the malignant biological behavior of the IGF2BPs gene family in HNSCC, the expression of the IGF2BP2 gene was knocked down in HNSCC cell line SCC4 cells using small interfering RNA. The effects of knocking down IGF2BP2 expression levels on the biological functions of the SCC4 cell line were analyzed using CCK8(cell counting kit-8)assay, EDU(5-Ethynyl-2′-deoxyuridine)assay, wound healing assay, and transwell migration and invasion assays. The findings of this study revealed that the IGF2BPs gene family may function as a possible diagnostic and predictive biomarker for HNSCC patients, and it exerts a tumor-promoting role in HNSCC. Therefore, the IGF2BPs gene family has the ability to be utilized as a promising biomarker and therapeutic target for the management of HNSCC in the future.

2. Material and methods

2.1. Expression of the IGF2BPs gene family in pan-cancer and HNSCC

A schematic diagram was employed to depict the study structure (Fig. 1). The expression profiles of the IGF2BPs gene family, along with the associated clinical data for 44 healthy control tissues and 502 HNSCC tumor samples, were acquired from the TCGA database. The gene expression data in HTSeq-FPKM RNAseq format were subjected to conversion into TPM and log 2 transformation. The ggplot2 package in R (v 3.6.3) was used to analyze and visualize the expression data of the IGF2BPs gene family in HNSCC and pan-cancer samples. The Wilcoxon rank-sum test was utilized for unpaired samples. In the case of paired sample analysis, if the sample satisfies the Shapiro-Wilk normality test ($p > 0.05$), the paired sample *t*-test was used, otherwise, the Wilcoxon signed rank test was performed. Furthermore, the cBioPortal database was used to get the three-dimensional (3D) protein structures of the IGF2BPs gene family. The HPA database was searched to retrieve the immunohistochemical staining images of the IGF2BPs gene family in healthy control tissues and HNSCC. Furthermore, the pROC package in R was used to produce receiver operating characteristic (ROC) curves and the ggplot2 package for visualization. A logistic regression model was used to evaluate the association between the clinical features (shown in Table 1) and the IGF2BPs gene family expression levels.

2.2. Prognostic value estimation of the IGF2BPs gene family in HNSCC

The IGF2BPs gene family in pan-cancer was mapped using the GEPIA2 database for both overall survival and disease-free survival. Three prognostic indicators were investigated to ascertain the prognostic significance of the IGF2BPs gene family in HNSCC: disease-specific survival, overall survival, and progress-free interval survival. Kaplan-Meier curves were generated using the R tool survminer.

Table 1
Results of logistic regression illustrated the correlation identified between the expression of the IGF2BPs gene family and clinical features.

Characteristics	Total (N)	IGF2BP1 Odds Ratio(OR)	P value	Total (N)	IGF2BP2 Odds Ratio(OR)	P value	Total (N)	IGF2BP3 Odds Ratio(OR)	P value
T stage (T3&T4 vs. T1&T2)	487	1.861 (1.282–2.714)	**0.001**	487	1.647 (1.136–2.396)	**0.009**	487	1.468 (1.014–2.131)	**0.043**
N stage (N1&N2&N3 vs. N0)	480	1.000 (0.699–1.431)	1.000	480	1.034 (0.723–1.479)	0.855	480	0.983 (0.687–1.407)	0.926
M stage (M1 vs. M0)	477	0.254 (0.013–1.734)	0.222	477	0.246 (0.013–1.677)	0.211	477	0.236 (0.012–1.607)	0.198
Clinical stage (Stage III&Stage IV vs. Stage I&Stage II)	488	1.498 (0.983–2.296)	0.062	488	1.414 (0.929–2.166)	0.108	488	1.564 (1.026–2.397)	**0.039**
Radiation therapy (Yes vs. No)	441	1.230 (0.831–1.822)	0.302	441	0.823 (0.556–1.218)	0.331	441	1.438 (0.970–2.138)	0.071
Primary therapy outcome (PR&CR vs. PD&SD)	418	1.417 (0.771–2.645)	0.265	418	0.753 (0.406–1.383)	0.362	418	1.314 (0.715–2.452)	0.382
Gender (Male vs. Female)	502	0.960 (0.646–1.426)	0.840	502	1.130 (0.761–1.681)	0.545	502	1.331 (0.896–1.984)	0.158
Race (Asian&Black or African American vs. White)	485	1.316 (0.757–2.314)	0.333	485	1.238 (0.712–2.168)	0.451	485	1.122 (0.645–1.959)	0.685
Age (>60 vs. ≤ 60)	501	1.164 (0.820–1.653)	0.397	501	0.845 (0.595–1.200)	0.348	501	1.092 (0.769–1.551)	0.624
Histologic grade (G3&G4 vs. G1&G2)	483	1.516 (1.003–2.305)	0.050	483	0.952 (0.630–1.437)	0.813	483	0.995 (0.658–1.502)	0.979
Smoker (Yes vs. No)	492	1.177 (0.771–1.801)	0.450	492	1.177 (0.771–1.801)	0.450	492	1.095 (0.717–1.674)	0.674
Alcohol history (Yes vs. No)	491	0.981 (0.671–1.433)	0.920	491	0.970 (0.664–1.418)	0.876	491	1.168 (0.800–1.708)	0.422
Lymphovascular invasion (Yes vs. No)	341	1.143 (0.733–1.785)	0.556	341	1.082 (0.694–1.690)	0.729	341	1.159 (0.744–1.808)	0.513
Lymph node neck dissection (Yes vs. No)	499	1.458 (0.922–2.324)	0.109	499	1.742 (1.097–2.795)	**0.020**	499	0.751 (0.473–1.186)	0.221

Furthermore, the Coxph function in R was employed for conducting univariate and multivariate Cox regression analyses to investigate the association between clinical variables and prognosis. The ggplot2 package in R was utilized to construct forest plots. The rms package and survival package in R were used to build nomogram plots and calibration plots.

2.3. Functional terms identification of the associated genes of the IGF2BPs gene family

The GeneMANIA website was used to develop a gene-gene interaction (GGI) network to examine the interactions between the IGF2BPs gene family and other genes. To further explore the genes that are positively and negatively associated with the IGF2BP gene family in HNSCC, a heatmap of the top 10 genes was produced using the R ggplot2 package. Subsequently, 1169 genes closely related to all three members of the IGF2BPs gene family were identified. P-adjustment values were used in the functional enrichment analysis. Using the cluster profile package in R, which assessed the GO terms (cellular components [CCs], biological processes [BPs], molecular functions [MFs]), and KEGG pathways, the biological function enrichment of these 1169 genes was examined [16]. Additionally, the cluster profile package in R was utilized for GSEA.

2.4. The link between the IGF2BPs gene family and tumor immunity in HNSCC

In order to examine the relationship between the IGF2BPs gene family and the tumor immune microenvironment (estimate score, immune score, and stromal score) in HNSCC, this study used the estimate package in R. Moreover, 24 tumor-specific immune infiltrating cells were identified by Professor Bindea, and the GSVA package in R was employed to investigate the association of the IGF2BPs gene family with these infiltrating cells in HNSCC [17,18]. Immune-stimulatory and immune-inhibitory genes that have already been found were taken into consideration for the purpose of better understanding the immunological regulation mechanisms of HNSCC [19]. The link between the IGF2BPs gene family and these immune-regulatory genes was examined using the Pearson correlation coefficient. The ggplot2 tool in R was used to create a heat map to display the above-mentioned outcomes.

2.5. Cell culture and transfection

The SCC4 cell line model is a widely used cell line in head and neck squamous cell carcinoma (HNSCC) research. The human HNSCC cell line SCC4 was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM (Gibco, USA) medium supplemented with 10 % fetal bovine serum(Gibco, USA) and 1 % penicillin-streptomycin(Gibco, USA) at 37 °C and 5 % CO2 in a cell culture incubator. To knock down the expression of the IGF2BP2 gene, small interfering RNA (siRNA) targeting IGF2BP2 was designed and synthesized by RiboBio Biologicals (Guangzhou, China), and transfected using Lipofectamine 3000. The siRNA sequences used for knockdown are as follows:

si-NC: sense strand: UUCUCCGAACGUGUCACGUTT, antisense strand: ACGUGACACGUUCGGAGAATT; si-IGF2BP2-1: sense strand: GGCAUCAGUUUGAGAACUATT, antisense strand: UAGUUCUCAAACUGAUGCCTT; si-IGF2BP2-2: sense strand: CGAAGAGAUUCCUCUGAAATT, anti Sense strand: UUUCAGAGGAAUCUCUUCGTT; si-IGF2BP2-3: sense strand: GAAGUGAUCGUCAGAAUUATT, antisense strand: UAAUUCUGACGAUCACUUCTT.

2.6. Real-time quantitative PCR(RT-qPCR)

Total RNA was extracted from the SCC4 cell line and purified using Trizol reagent (Invitrogen, USA), followed by an evaluation of purity and integrity. The cDNA was then synthesized using a cDNA synthesis kit (Vazyme Bio, China) and subjected to real-time quantitative PCR analysis with the aid of the SYBR Green qPCR SuperMix kit (Vazyme Bio, China). The reaction conditions were as follows: 95 °C for 5 min, 95 °C for 15 s, and 60 °C for 34 s, for a total of 40 cycles. The primer sequences required for the experiment were as follows: IGF2BP2:F- GACGTCAGCGAAAGGATGGT, R- GCGCTTCCAGCTTCACTTCT; 18S: F- CCTGGATACCGCAGCTAGGA, R-GCGGCGCAATACGAATGCCCC. The internal reference used was 18S, and relative expression levels were calculated using the $2^{-\triangle\triangle CT}$ method.

2.7. Western blot

Total protein was isolated from the SCC4 cell line using a complete protein extraction kit supplied by KeyGene Bio (China), and protein concentration was measured using a BCA kit (KeyGene Bio, China). Proteins were separated using 10 % SDS-PAGE and then transferred onto PVDF membranes (Beyoime Bio, China). PVDF membranes were subjected to incubation with primary antibodies IGF2BP2 (ab129071, Abcam, 1:1000) and GAPDH (ab128915, Abcam, 1:10000) at 4 °C overnight. The membranes were washed using PBS the following day, and the membranes were incubated with secondary antibodies for 1 h at room temperature. The exposure process was then carried out using an ECL developer (Beyoime Bio, China).

2.8. CCK8(cell counting kit-8) assay

The SCC4 cells of the si-NC group and the si-IGF2BP2-3 group were cultured routinely. The two sets of cells that had good growth status were chosen, and they were inoculated into a 96-well plate at a density of 3×10^3 cells per well. The cells were collected for 1–3 days, and 10 μl of CCK8 reagent (KeyGene Bio, China) was added to each well under light-proof conditions. Following a 1-h incubation

at 37 °C, the absorbance (OD) value at 450 nm was determined using an enzyme marker.

2.9. EDU(5-ethynyl-2′-deoxyuridine) assay

The above two groups of cells in good growth condition were selected and inoculated in 96-well plates at 3×10^3 cells per well. After reaching the log phase, 100 μl of EDU (Beyoime Bio, China) solution was added. The mixture was then incubated for 3 h at 37 °C and fixed for 20 min with 4 % paraformaldehyde. Then, glycine and PBS containing 0.5 % Triton X-100 were used to wash the cells. The cells were incubated for 30 min after adding 100 μl of ApolloR solution per well (protected from light). The cells were then subjected to an additional 30 min of incubation after Hoechst (100 μl) was added to each well. Finally, using a fluorescence microscope, the cells were photographed and counted.

2.10. Wound healing assay

First, the transfected si-NC and si-IGF2BP2-3 two groups of SCC4 cell lines were cultured in 6-well plates at a density of 1×10^6 with

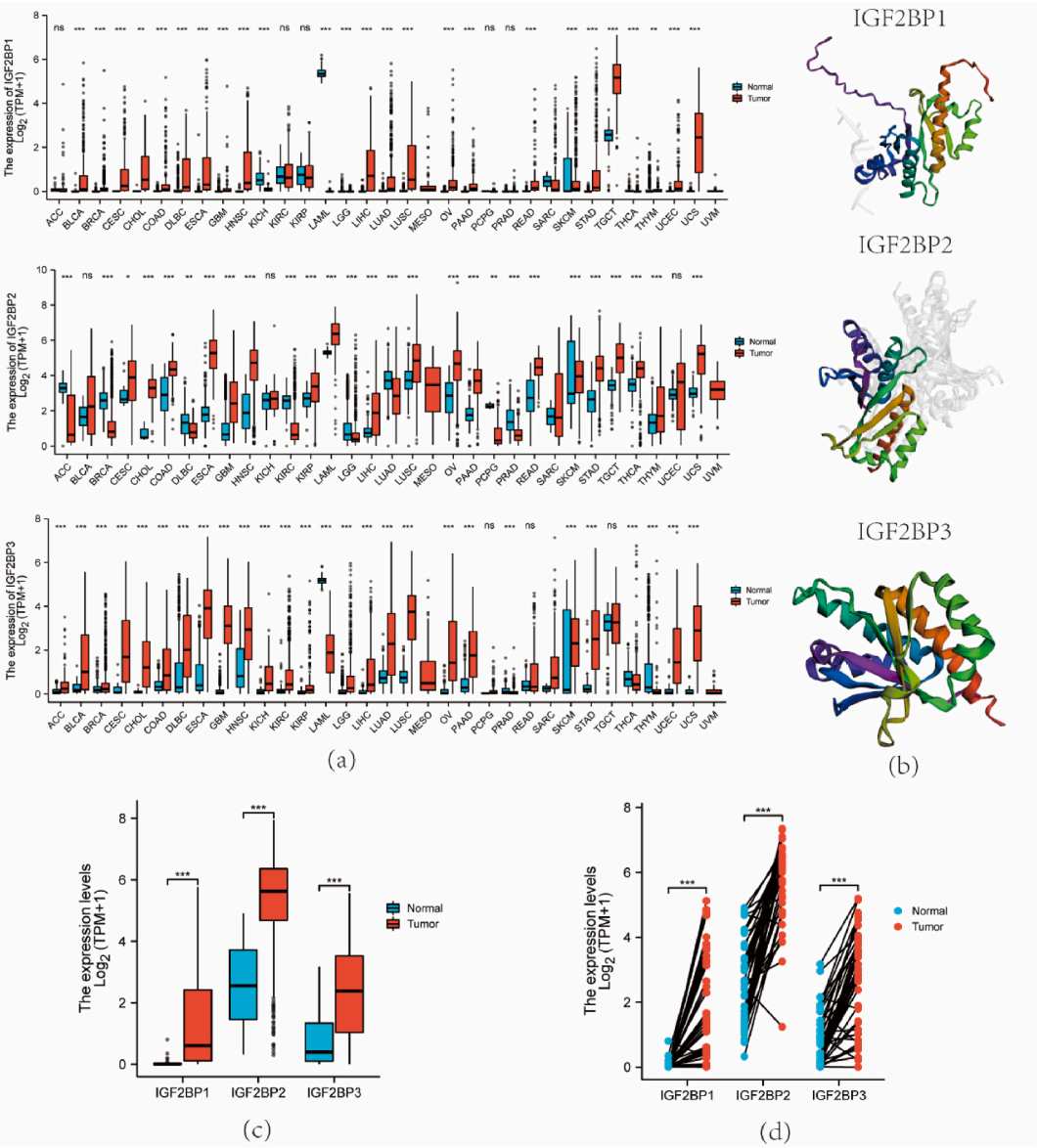

Fig. 2. The pattern of mRNA expression of the IGF2BPs gene family in pan-cancer and HNSCC. (a) The pattern of mRNA expression of the IGF2BPs gene family in pan-cancer in TCGA database. (b) The 3D protein structure of the IGF2BPs gene family. (c–d) The pattern of mRNA expression of the IGF2BPs gene family in HNSCC in TCGA database using paired and unpaired sample analysis.

complete medium. Cultivate until the next day, and when the cell density is >90 % as observed under a microscope, use a sterile 200 μl pipette tip to scratch perpendicularly to the plane of the 6-well plate, and wash with PBS three times to remove the fallen cells. After that, the serum-free medium was replaced to continue the culture, and the same scratch position was photographed at 0 h and 24 h. The experiment was repeated 3 times, and Image J was used to analyze the scratch width.

2.11. Transwell migration and invasion assay

The SCC4 cells of the si-NC group and si-IGF2BP2-3 group were cultured until good growth status was achieved. These cells were then inoculated into transwell chambers (Corning Incorporated, USA) at a density of 3×10^4 per well for the migration assay. In the upper chamber, a serum-free medium was introduced, and in the lower chamber, a complete medium was added. Following a 24-h incubation period, the transwell chambers were removed. Then, the cells were fixed for 20 min in 4 % paraformaldehyde and stained in 0.1 % crystal violet. The cells were subsequently photographed and counted under a microscope. In the invasion assay, Matrigel gel (Corning Incorporated, USA) was applied to the transwell chambers in advance.

2.12. Statistical methods

For the in vitro assays, all experiments were performed in three biological replicates. Data were collected using Image J and statistically analyzed on GraphPad Prism 8 using a *t*-test with two independent samples and one-way variance. The threshold for statistical significance was set at $P < 0.05$. ***$p < 0.001$; **$p < 0.01$; and *$p < 0.05$.

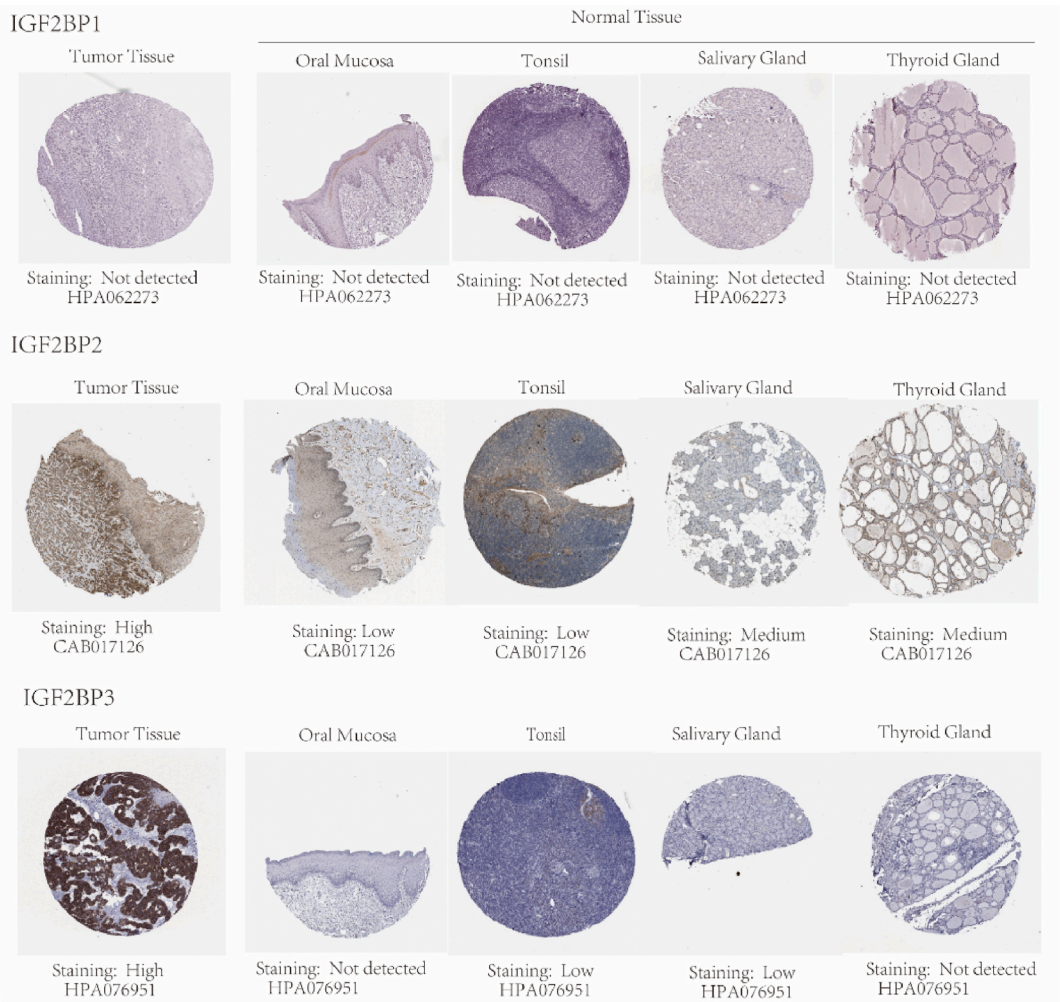

Fig. 3. The protein expression level of the IGF2BPs gene family in HNSCC and healthy control tissues in the HPA database. Immunohistochemical profiles showed the protein level of expression of the IGF2BPs gene family in HNSCC with significantly higher expression compared to healthy tissues.

3. Results

3.1. The expression profile of IGF2BPs gene family in HNSCC

The expression pattern of all members of the IGF2BPs gene family across pan-cancer was examined (Fig. 2a). Significant upregulation of IGF2BP1, IGF2BP2, and IGF2BP3 was observed in several malignancies, including BRCA, CESC, CHOL, ESCA, LIHC, COAD, LUSC, HNSC, GBM, PAAD, READ, OV, SKCM, UCEC, STAD, and UCS. The mRNA expression levels of the IGF2BPs gene family were found to be considerably higher in HNSCC samples as compared to healthy oral samples ($P < 0.001$), as revealed by both unpaired and paired sample analyses (Fig. 2c and d). The same results were observed in the GEO database (Fig. S2 a-d).

Furthermore, the 3D protein structures of three genes from the IGF2BPs gene family were visualized (Fig. 2b). The protein expression levels of the IGF2BPs gene family in both healthy and cancerous tissues were also assessed from the HPA database (Fig. 3). The findings showed that the IGF2BP2 and IGF2BP3 protein levels were elevated in HNSCC tissues but lowered in normal tissues, including oral mucosa, tonsils, salivary gland, and thyroid gland. Nevertheless, IGF2BP1 was not observed to be expressed in either HNSCC or healthy tissues. In addition, the protein expression level of the IGF2BPs gene family in HNSCC was higher than in normal tissues according to the UALCAN portal(Fig. S2 h-j).

Additionally, to evaluate the diagnostic usefulness of the IGF2BPs gene family in HNSCC, the ROC curve was employed. The variables of IGF2BP1, IGF2BP2, and IGF2BP3 revealed a higher accuracy in distinguishing healthy controls from HNSCC samples (AUC = 0.929, 0.910, and 0.811.) (Fig. 4a–c).

3.2. Correlation between clinicopathologic features and expression of the IGF2BPs gene family in HNSCC

The study utilized logistic regression analysis to investigate the association between the clinical characteristics of patients with HNSCC and the mRNA expression of genes in the IGF2BPs gene family (Table 1). The findings revealed a positive association of IGF2BP1 expression with the T stage ($P = 0.001$). IGF2BP2 expression was also significantly positively correlated with lymph node neck dissection and T stage ($P = 0.020$ and $P = 0.009$, respectively). Moreover, there was a positive association of IGF2BP3 expression with the clinical stage ($P = 0.039$) and T stage ($P = 0.043$).

3.3. Significance of the IGF2BPs gene family in the prognosis of HNSCC

The survival heatmaps (Fig. 5a and b) depict the prognostic significance of the IGF2BPs gene family for overall survival and disease-free survival in diverse tumor types.

The Kaplan–Meier curves(Fig. 5c) revealed that HNSCC patients with elevated expression levels of IGF2BP2 displayed a lower overall survival(OS) ($p = 0.006$), with no considerable link to progression-free interval survival (PFI) and disease-specific survival (DSS). The upregulation of IGF2BP2 was related to a poorer HNSCC prognosis, however, the IGF2BP1 and IGF2BP3 expressions did not reveal any links to DSS, PFI, and OS. The same results were observed in the GEO database (Fig. S2 e-g).

The univariate analyses demonstrated that certain clinical characteristics were significant risk factors for mortality in individuals with HNSCC (Fig. 6a). The M1 stage ($P = 0.002$), the lack of radiation therapy ($P = 0.002$), the SD and PD primary therapy outcomes ($P < 0.001$), and lymphovascular invasion ($P = 0.002$) were among these. Importantly, the HR value of 1.385 for the high expression level of IGF2BP2 in individuals with HNSCC illustrated that the risk of death increased in this group in comparison to the group with low expression ($P = 0.018$). The multivariate analyses (Fig. 6b) showed that a number of variables, such as high N stage (N1, N2, and

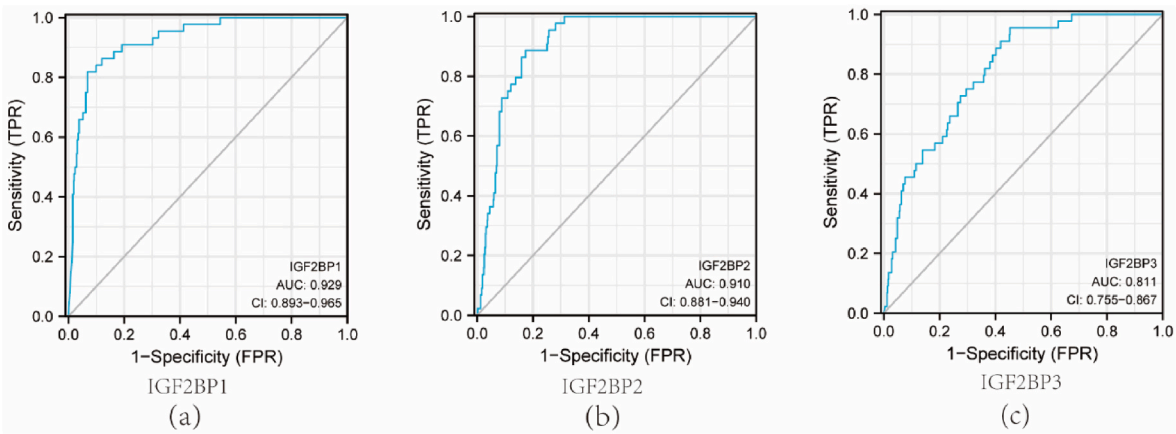

Fig. 4. The diagnostic values of the IGF2BPs gene family in HNSCC were evaluated using ROC curves. The AUC area of the ROC curve above 0.9 represents a high accuracy in predicting HNSCC. Fig. 4(a–c) shows that the diagnostic values of the IGF2BPs gene family in HNSCC all have high accuracy.

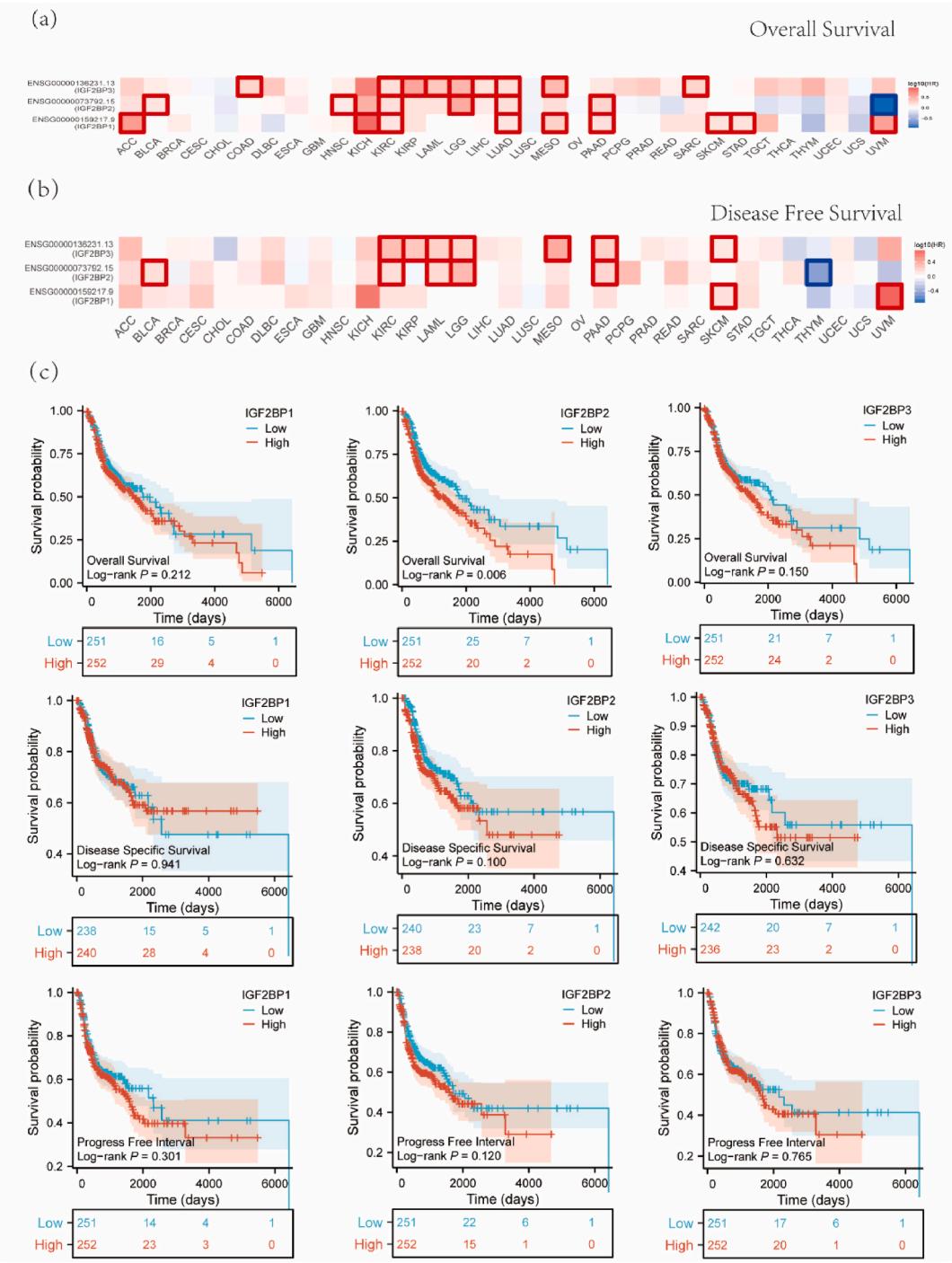

Fig. 5. Survival analysis findings of the IGF2BPs gene family in pan-cancer and HNSCC. (a–b) The survival map illustrated the prognosis-predictive values of the IGF2BPs gene family in overall survival (OS) and disease-free survival (DFS) of multiple pan-cancer. (c) The IGF2BPs gene family and three prognostic outcomes—disease-specific survival (DSS), progression-free interval survival (PFI), and overall survival (OS) in individuals with HNSCC—were correlated, as shown by the Kaplan-Meier curves.

N3) ($P = 0.047$), lack of radiation therapy, and primary therapeutic outcomes of SD and PD ($P < 0.001$), had an impact on overall survival in individuals with HNSCC. However, the level of IGF2BP2 expression did not have an impact on survival outcomes.

Moreover, to illustrate the connection between the IGF2BPs gene family and survival likelihood, a nomogram was created (Fig. 7a). The individuals with higher points exhibited poor survival outcomes. Additionally, the calibration curves demonstrated that the

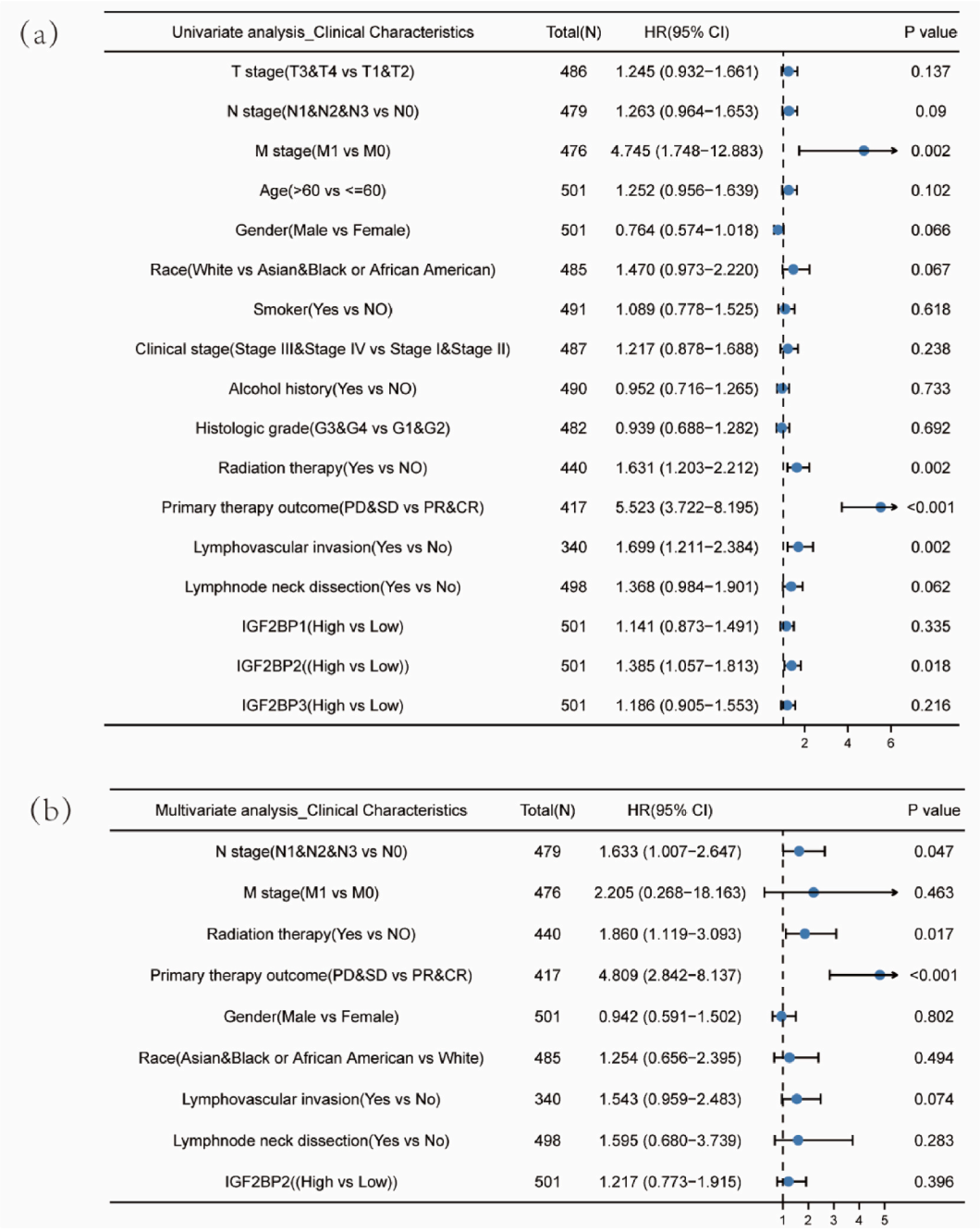

Fig. 6. Forrest plots revealed the correlation between the clinical features and the IGF2BPs gene family in HNSCC. (a) The Univariate Cox regression analyses. (b) The multivariate Cox regression analyses.

nomogram plot exhibited excellent predictive ability for OS over 1 and 3 years (Fig. 7b).

3.4. The associated and Co-expressed genes of the IGF2BPs gene family

A GGI network of the IGF2BPs gene family was generated using the STRING database (Fig. 8a). Furthermore, the top 20 co-expressed genes were considered crucial, highly connected hub node genes, i.e., ELAVL4, MOV10, YBX1, and MYC, performing a crucial role in some important biological functions, for instance, negative regulation of cellular amide metabolic process, mRNA catabolic process regulation, and RNA stability regulation.

The ten leading genes of the IGF2BPs gene family with the highest levels of positive correlation, including PLAG1, HMGA2, and

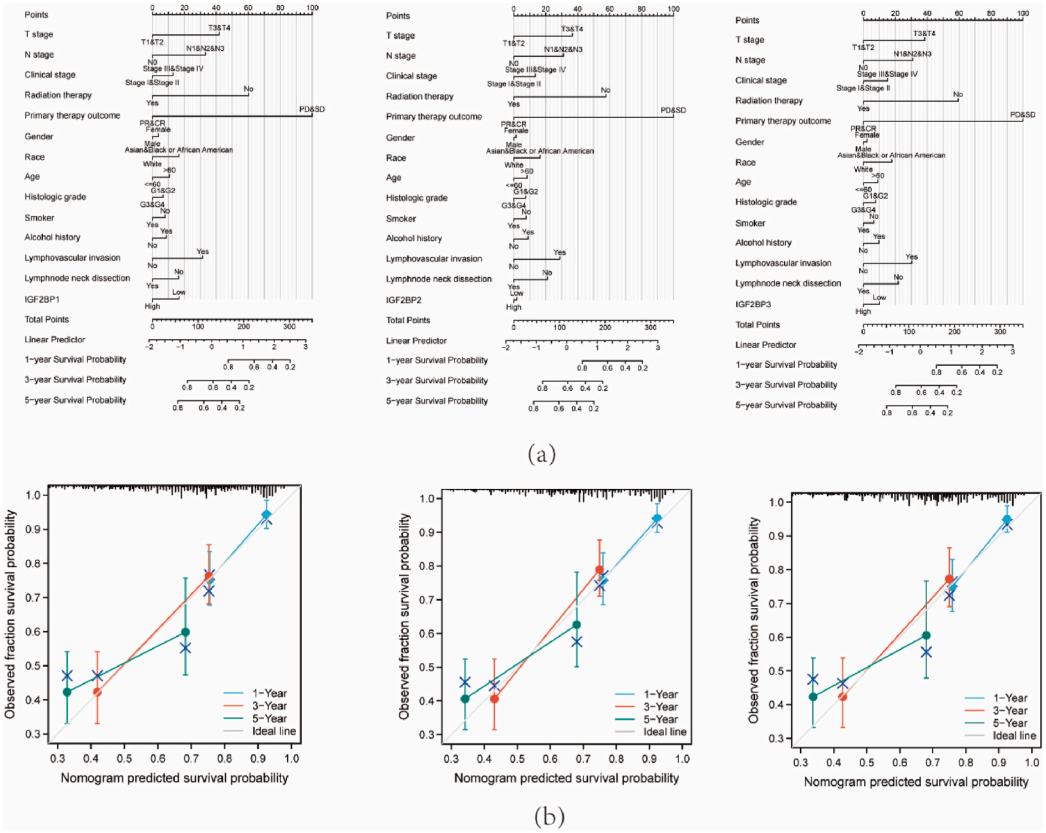

Fig. 7. The nomogram and calibration plots. (a) The nomogram plots were utilized to assess the OS over 1, 3, and 5 years for HNSCC patients. (C) The calibration plots were utilized to predict the accuracy of the nomogram model of 1, 3, and 5- years.

DDX18, and the top ten genes with the lowest levels of expression, including S100A8, TNFRSF13B, and CST3, were illustrated on a heatmap (Fig. 8b).

3.5. Biological function enrichment analysis of the IGF2BPs gene family in HNSCC

The 1169 correlated genes of the IGF2BPs gene family that were selected (Fig. 9a) were subjected to GO analysis, KEGG analysis, and gene set enrichment analysis. Bubble charts (Fig. 9b–e) were generated to represent the analysis results of GO terms, including cellular components (CC), biological processes (BP), and molecular functions (MF), along with KEGG pathway analysis results. Additionally, the mountain plot (Fig. 9f) demonstrated the findings of gene set enrichment analysis (GSEA).

Furthermore, the substantially linked genes of the IGF2BPs gene family were predominantly enriched in some specific biological processes, for instance, proteasomal protein catabolic process, regulation of neuron projection development, and process utilizing autophagic mechanism (Fig. 9b). The substantially associated genes of the IGF2BPs gene family were predominantly enriched in certain cellular components (Fig. 9c), such as the cell leading edge, cell−substrate junction, and focal adhesion. The considerably related genes of the IGF2BPs gene family were mainly enriched in certain molecular functions, i.e., cell adhesion molecule binding, serine/threonine protein kinase activity, and cadherin binding (Fig. 9d). The associated genes were primarily linked to the regulation of several cellular processes, including the actin cytoskeleton, endocytosis, focal adhesion, protein processing in the endoplasmic reticulum, and signaling pathways like the Hippo and Wnt pathways, as well as pathogenic Escherichia coli infection, according to the KEGG enrichment analysis (Fig. 9e).

In addition, the results of GSEA illustrated that substantial enrichment of these associated genes was observed in various signaling pathways, i.e., focal adhesion, PI3K-AKT and MAPK signaling pathways, and epithelial-mesenchymal transition (Fig. 9f).

3.6. The correlation between the IGF2BPs gene family and tumor immunity in HNSCC

The estimate-immune-stromal score was used to assess the relationship between the expression of the IGF2BPs gene family and the tumor immune microenvironment (Fig. 10). The scatter plot (Fig. 10g–i) revealed a considerable negative association between the immune score and the expression of IGF2BP1 ($r = -0.176$; $p < 0.001$), IGF2BP2 ($r = -0.335$; $p < 0.001$), and IGF2BP3 ($r = -0.242$; $p < 0.001$). Furthermore, the estimate score (Fig. 10a–c) was found to be significantly negatively associated with the expression of

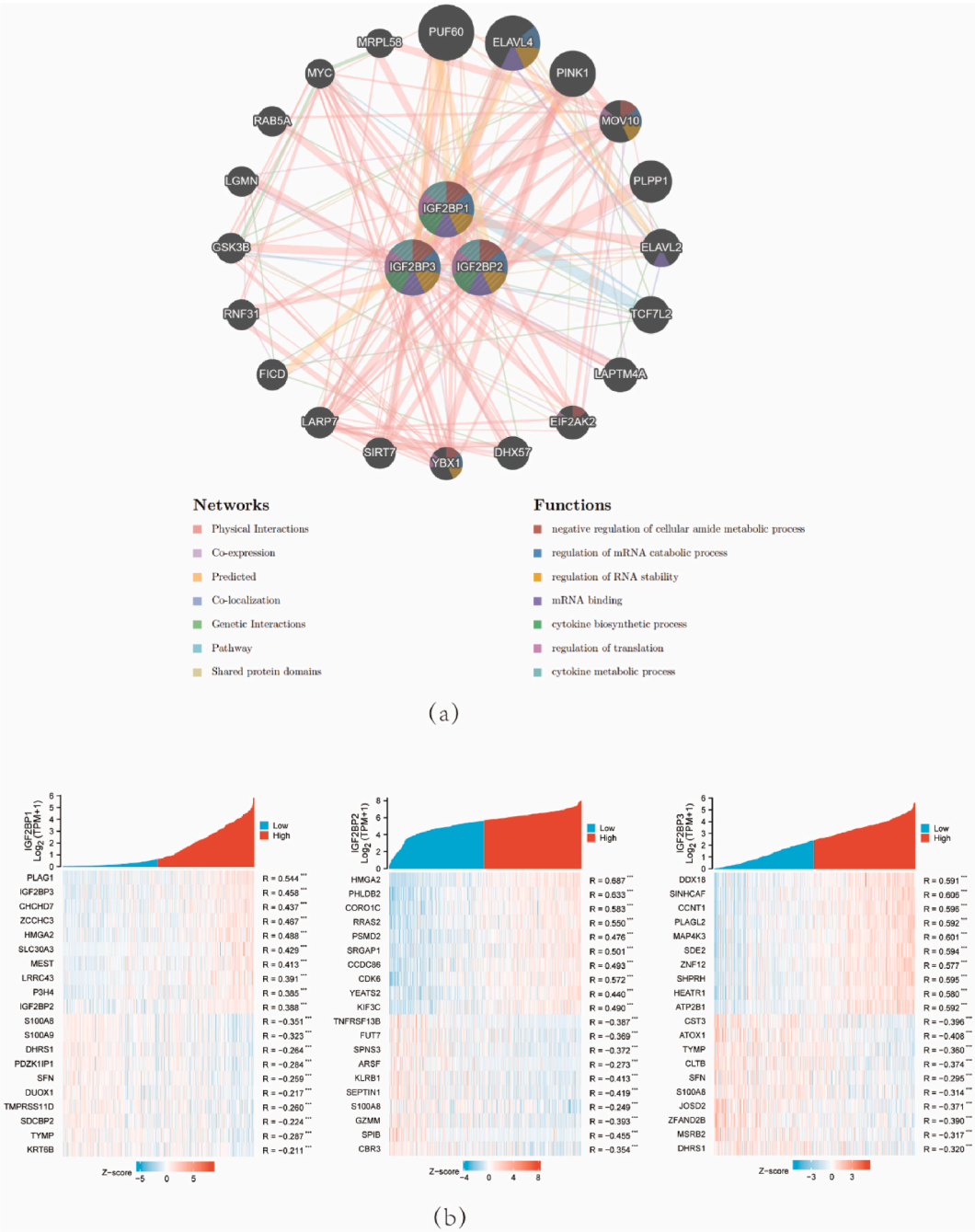

Fig. 8. The co-expressed and associated genes of the IGF2BPs gene family. (a) Gene-gene interaction (GGI) network. (b) Top 10 genes positively and negatively correlated with IGF2BPs gene family in HNSCC.

IGF2BP1 ($r = -0.092$; $p = 0.040$), IGF2BP2 ($r = -0.235$; $p < 0.001$)), and IGF2BP3 ($r = -0.151$; $p < 0.001$). However, no significant link was found between the expression of the IGF2BPs gene family and stromal score(Fig. 10d–f).

Moreover, as per the Pearson correlation study, tumor immune infiltration cells (TIICs) displayed a negative association with the expression of the IGF2BPs gene family, such as T cells, Th17 cells, and Treg (Fig. 11a).

The study also examined the relationship between the IGF2BPs gene family and genes that promote or suppress the immune system in HNSCC. Immunoinhibitory genes such as NECTIN2, TGFBR1, and VTCN1 were found to correlate significantly with the expression of the IGF2BPs gene family. Immunostimulatory genes such as TMIGD2, TNFRSF13B, and TNFRSF4 were significantly negatively associated with the expression of the IGF2BPs gene family (Fig. 11b).

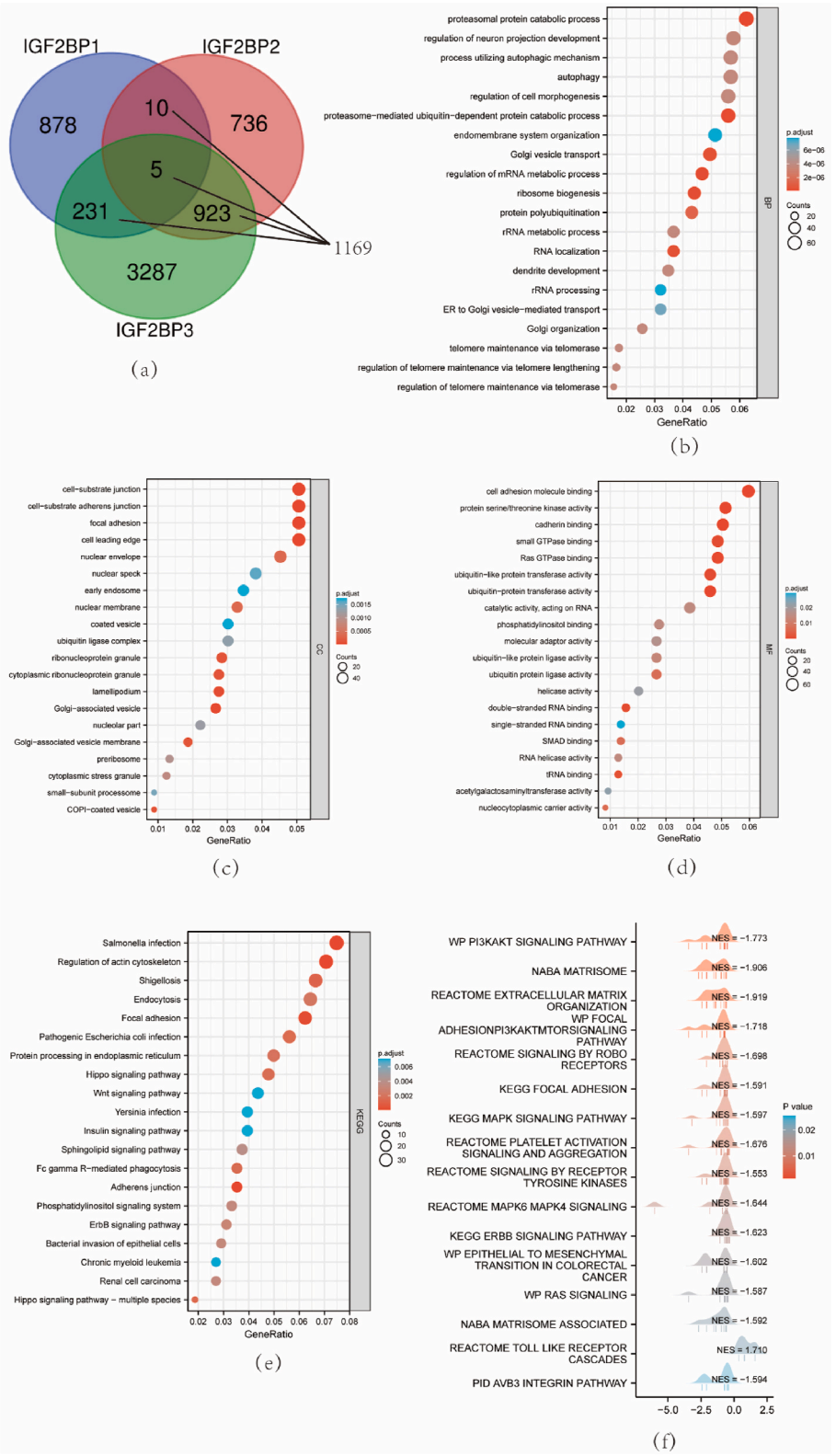

Fig. 9. Biological function enrichment analysis. (a) The Venn diagram illustrated the associated genes in the IGF2BPs gene family. (b) The biological processes (BP) in GO terms. (c) The cellular components (CC) in GO terms. (d) The molecular functions (MF) in GO terms. (e) The KEGG signaling pathways. (f) The gene set enrichment analysis.

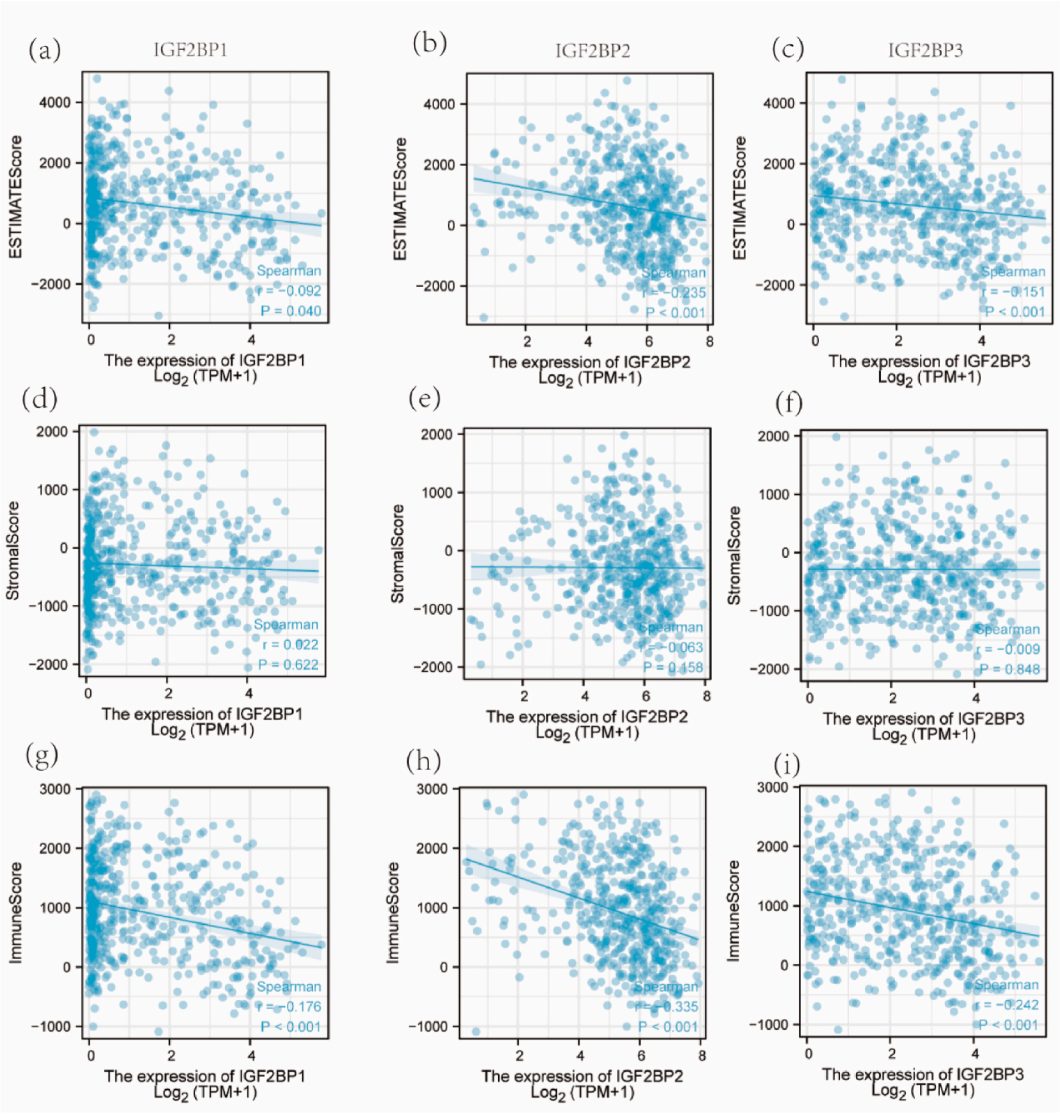

Fig. 10. The link between the IGF2BPs gene family and tumor microenvironment by evaluation of the Estimate-Immune-Stromal score in HNSCC. (a–c) Scatter plots revealed a significant negative correlation between the IGF2BPs gene family and the estimate score. (d–f) Scatter plots showed that there is no significant correlation between the IGF2BPs gene family and the stromal score. (g–i) Scatter plot illustrated the obvious negative correlation between IGF2BPs gene family and immune score.

3.7. Knockdown of IGF2BP2 gene expression inhibited SCC4 cell line proliferation, migration, and invasion

To gain further insights into the potential involvement of the IGF2BPs gene family in the malignant progression of HNSCC, the IGF2BP2 gene was subjected to in vitro experiments. Through Western blot experiments (Fig. 12a), it was found that IGF2BP2 is highly expressed in HNSCC cell lines (SCC4, CAL27) relative to non-malignant oral cells (NOK). We chosed the SCC4 cell line for the next functional experiment. To achieve knockdown of IGF2BP2 expression in the SCC4 cell line, siRNA was employed, and the knockdown efficiency was validated by RT-qPCR and Western blot assays (Fig. 12b and c). The si-IGF2BP2-3 effectively attenuated the expression of the IGF2BP2 gene in the SCC4 cell line when compared to si-NC. Thus, si-IGF2BP2-3 was selected for further experiments. The proliferation ability of the SCC4 cell line was evaluated using CCK8 and EDU assays (Fig. 12d and e). The knockdown of the IGF2BP2 gene through siRNA significantly suppressed the DNA replication and proliferation capabilities of the SCC4 cell line when compared to the si-NC treatment. Additionally, the invasion and migration capabilities of the SCC4 cell line were evaluated through wound healing assay and transwell assay (Fig. 12f and g), which showed that the knockdown of the IGF2BP2 gene could effectively suppress the cell migration and invasion abilities.

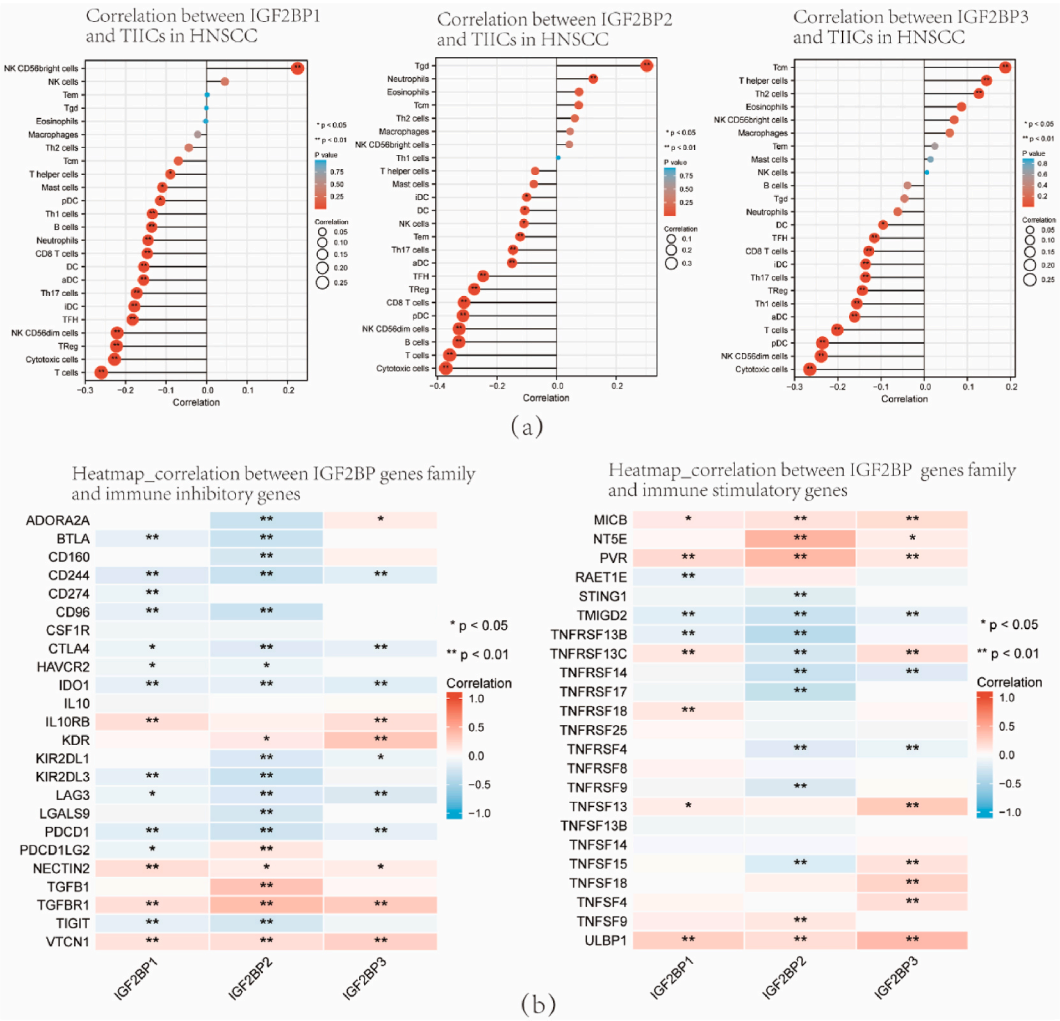

Fig. 11. The link between the IGF2BPs gene family and tumor immunity in HNSCC. (a) The link between immune cell infiltration and the IGF2BPs gene family in HNSCC. (b) The link between immune inhibitory/stimulatory genes and the IGF2BPs gene family in HNSCC.

4. Discussion

Despite significant advancements in the treatment of HNSCC, including surgery, radiotherapy, and chemotherapy, the disease remains challenging due to its propensity for metastasis and recurrence. Even though immunotherapy and targeted therapy have emerged as promising treatments for HNSCC in recent years, drug resistance remains a major issue [20]. The IGF2BPs gene family is an RNA-binding protein containing IGF2BP1, IGF2BP2, and IGF2BP3. Previously reported studies have demonstrated that the IGF2BPs gene family is aberrantly expressed in lung, liver, and colorectal cancers and associated with poor prognosis, but its prognostic value and potential biological function in HNSCC have not been revealed [7–14]. This study creatively combined bioinformatics analysis with in vitro experiments to reveal the important role of the IGF2BPs gene family in HNSCC and provide a basis for future therapeutic strategies for HNSCC.

In this study, the expression level of the IGF2BPs gene family was shown to be significantly higher in HNSCC than in healthy head and neck tissues. Previous studies also showed that IGF2BP2 and IGF2BP3 expression levels were significantly greater in HNSCC tumor tissues compared to healthy control samples, and they were linked to a poor prognosis in HNSCC patients [21,22]. Furthermore, the present investigation revealed that the elevated expression level of the IGF2BPs gene family was linked to the clinical stage, T stage, and lymph node neck dissection. Lin and Young et al. also showed that IGF2BP2 and IGF2BP3 in HNSCC were strongly associated with cancer stage and lymph node metastasis in HNSCC [23,24]. The findings suggest that overexpression of the IGF2BPs gene family may promote the onset and progression of HNSCC by enhancing lymph node metastasis.

Furthermore, through the analysis of ROC curves, it was observed that the IGF2BPs gene family had a high accuracy in distinguishing between HNSCC samples and normal control tissues. In studying preoperative biopsy material from HNSCC patients, Achille et al. studied that IGF2BP3 was closely associated with perineural infiltration in HNSCC patients and could assist in the

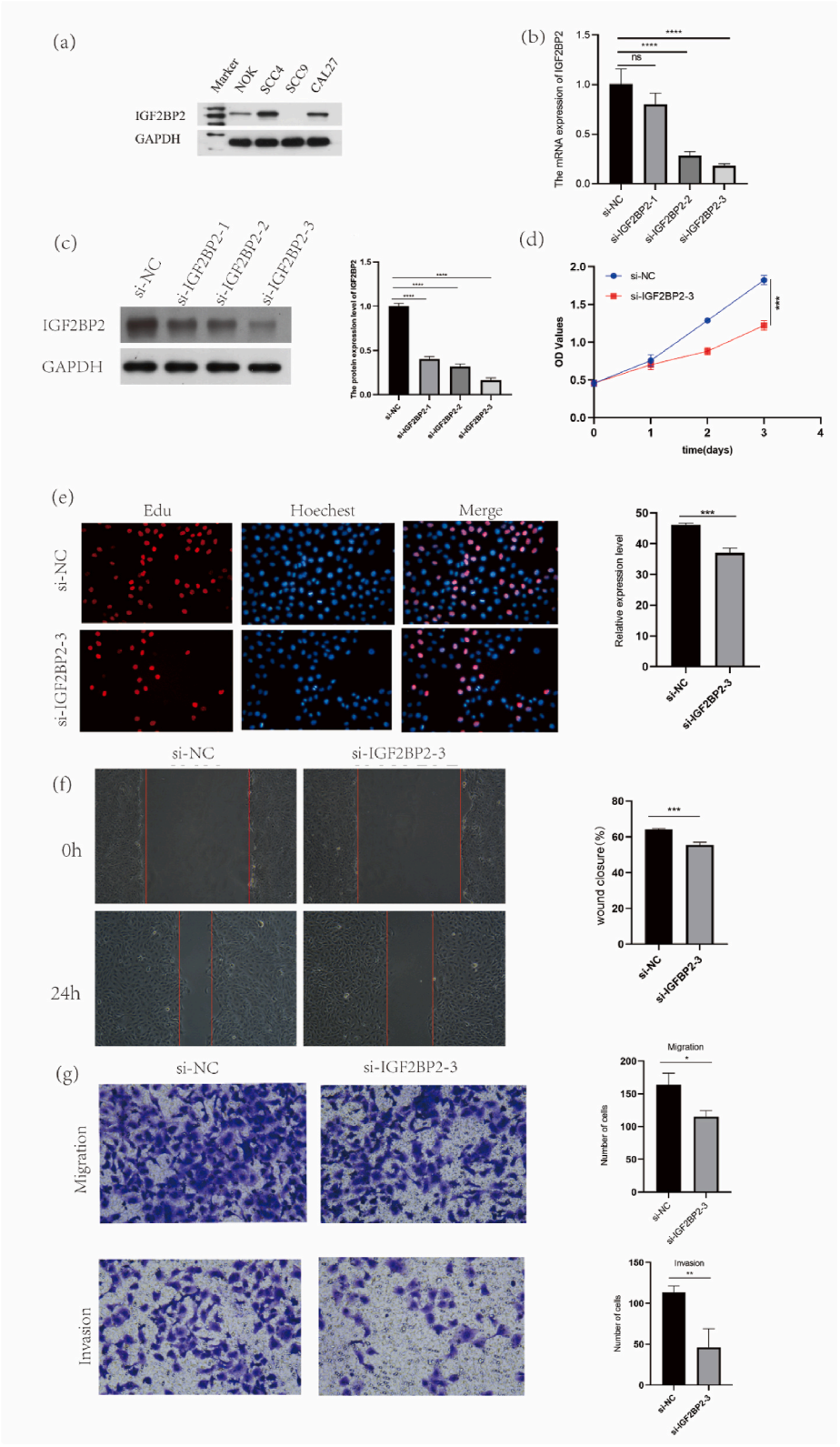

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Fig. 12. The proliferation, migration, and invasion capacities of the SCC4 cell line were inhibited by the knockdown of IGF2BP2 expression. (a) IGF2BP2 is highly expressed in HNSCC cell lines relative to non-malignant oral cells (HOK). (b–c) The expression of IGF2BP2 in the SCC4 cell line was knocked down by siRNA and the knockdown efficiency was verified by RT-qPCR and Western blot assays. (d–e) CCK8 and EDU proliferation assays revealed that the knockdown of IGF2BP2 expression considerably inhibited the proliferation behavior in SCC4 cell lines compared to si-NC. (f) Wound healing assay demonstrated that knockdown of IGF2BP2 expression significantly inhibited migration behavior in the SCC4 cell line compared to si-NC. (g) Transwell assay showed that knockdown of IGF2BP2 expression significantly inhibited migration and invasion behavior in the SCC4 cell line compared to si-NC. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

diagnosis of HNSCC patients and perform accurate preoperative stratification, leading to accurate treatment planning [25]. This evidence suggested that the IGF2BPs gene family may be a diagnostic biomarker for HNSCC.

By comparing patients with high IGF2BP2 expression to those with low expression, the examination of patient survival data showed a substantial decline in OS. Univariate regression analysis confirmed IGF2BP2 as an independent risk factor for HNSCC, which is consistent with previous studies [23,26]. The association between higher IGF2BP2 expression and worse survival results raises the possibility that IGF2BP2 could be used as an independent biomarker to predict prognosis in HNSCC patients. Based on the combined expression levels of the IGF2BPs gene family and other clinical parameters for predicting 1-, 3-, and 5-year survival, a nomogram plot was created to improve the accuracy of survival prediction. To validate these results, however, additional clinical research with larger cohorts is required.

The GGI network revealed co-expression of the genes of the IGF2BPs gene family with some other important genes, i.e., YBX1, which colocalized with IGF2BP1 in the cytoplasm. IGF2BP1 stabilized the YBX1 by correlating with the Coding Region instability Determinant (CRD) [27]. Moreover, YBX1 was upregulated in lung adenocarcinoma and acted as an independent predictor of the prognosis and recurrence of lung adenocarcinoma [28]. The stabilization of YBX1 by IGF2BP1 may be a crucial mechanism in the onset and progression of the disease. The GGI network also showed that the MYC gene is co-expressed with the IGF2BPs gene family. MYC has been proven to be an oncogenic gene in many tumors, and its high expression and deregulation are associated with poor prognosis in tumor patients. In a previous study, it was shown that IGF2BP2 can interact with long non-coding RNA HOXB-AS3, thereby increasing the stability of the MYC gene to promote the proliferation activity of oral squamous cell carcinoma cells [29]. These findings suggest that myc may be a downstream gene of the IGF2BPs gene family, and the IGF2BPs gene family may interact with MYC to promote downstream signal transduction to promote the malignant behavior of HNSCC cells.

The IGF2BPs gene family was found to be enriched in several key biological processes, such as the PI3K-AKT, and MAPK signaling pathways, as well as epithelial-mesenchymal transition. In colorectal cancer, METTL3 targeted EphA2 and VEGFA via different IGF2BPs-dependent mechanisms were found to promote vasculogenic mimicry formation via PI3K/AKT/mTOR signaling [30]. The levels of expression of IGF2BP3 were considerably elevated in Glioblastoma in comparison to healthy tissues. The effects of IGF2BP3 were exerted on Glioblastoma through IGF-2, leading to the activation of MAPK signaling, which eventually promoted the progression of Glioblastoma [31]. CircIGHG was significantly elevated in HNSCC and associated with poor prognosis. CircIGHG could target miR-142–5p to enhance IGF2BP3 activity and promote HNSCC progression through the epithelial-mesenchymal transition [22]. In conclusion, the IGF2BPs gene family may promote tumor development and metastasis through various pathways including the PI3K-AKT signaling pathway, MAPK signaling pathway, and epithelial-mesenchymal transition.

Tumor immunotherapy has long been approved for patients with recurrent and metastatic HNSCC. The tumor immune microenvironment has been shown to be potentially involved in the response to tumor immunotherapy [32]. It was found that the IGF2BPs gene family was negatively associated with the tumor immune microenvironment in HNSCC. Yang et al. conducted a study demonstrating the close association of IGF2BP1 with the immune microenvironment in hepatocellular carcinoma. Their findings revealed that IGF2BP1 knockdown considerably increased the infiltration of immune cells such as T cells, NK cells, and macrophages in tumors and decreased the expression of PD-L1. Hence, IGF2BP1 could hold value as a target for immunotherapy in patients with hepatocellular carcinoma [33]. As a result of the suppressed tumor immune microenvironment, these findings show that high expression of the IGF2BPs gene family may promote tumor advancement.

Additionally, the study revealed that the IGF2BPs gene family has a strong association with T cells and other immune infiltrating cells, which are vital in anti-tumor activities during immunotherapy. Exhausted T cells are known to contribute to uncontrollable tumor growth [34]. Wan et al. demonstrated that inhibiting IGF2BP3 expression in breast cancer leads to T cell activation, thereby improving immune regulation in tumors [35]. These findings indicated that high expression of the IGF2BPs gene family may lead to accelerated tumor progression by inhibiting tumor immune infiltrating cells. In addition, it was also revealed that the IGF2BPs gene family was substantially associated with quite a few immune inhibitory genes, i.e., TGFB1, and some immune-stimulatory genes, i.e., TNFRSF4. Cancer-associated fibroblasts (CAFs) within the tumor microenvironment in HNSCC contributed to promoting immunosuppression and evasion from immune surveillance. Moreover, CAFs had a greater TGFB1 expression level [36]. These investigations suggested that the IGF2BPs gene family can accelerate the development of HNSCC by controlling immune inhibitory genes. Lower levels of expression of TNFRSF4 were linked to poor survival, and it can play a key role in HNSCC outcomes [37]. The IGF2BPs gene family can stimulate the development of HNSCC by attenuating the expression of these immune-stimulatory genes. In conclusion, the IGF2BPs gene family has a close association with tumor immunity in HNSCC, suggesting that further research on this gene family may provide a new foundation for the development of immunotherapy for HNSCC.

Finally, in vitro research was conducted to determine how the IGF2BPs gene family affects the biological functioning of HNSCC. IGF2BP2 expression was knocked down in the HNSCC cell line SCC4, and several functional tests were carried out to investigate the function of IGF2BP2 in HNSCC. After knocking down the expression level of IGF2BP2, the migration, cell proliferation, and invasion abilities of SCC4 were found to have considerably reduced. The outcomes of Ke et al. were consistent with the current study, where a

considerable attenuation in the proliferation and migration abilities of HNSCC cells was studied following a knockdown of IGF2BP2 expression [21]. These results imply that IGF2BP2 might play a significant role in the development of HNSCC and may act as a future therapeutic target for HNSCC treatment.

The comprehensive nature of the bioinformatics analysis - integrating expression, diagnostic value, prognostic value, co-expression analysis, pathway analysis, and immune associations provides a 360-degree view. The objective of the current investigation was to examine the expression pattern and diagnostic and prognostic significance of the IGF2BPs gene family in HNSCC, along with investigating its relationship with tumor immunity to enhance comprehension of its regulation of the tumor microenvironment. Our bioinformatics analysis of large patient cohorts exceeds 500 HNSCC samples. This lends statistical power to the clinical findings. In addition, it was verified by in vitro experiments that IGF2BP2 could promote HNSCC development. In this study, a combination of bioinformatics and in vitro approach provides both clinical and functional evidence on the IGF2BPs gene family in HNSCC. Nonetheless, there are several shortcomings in the study that must be addressed. Additional research is required to validate the biological significance of the IGF2BPs gene family in HNSCC and clarify the underlying mechanisms. For example, functional analysis is restricted to one HNSCC cell line. Expanding the number of cell line models would improve robustness. Adding vivo animal model experiments will help demonstrate IGF2BPs gene family's functional role in promoting HNSCC progression. Secondly, the prognostic role of the IGF2BPs gene family in HNSCC needs to be further validated by clinical large cohort studies. Finally, more research on the link between HNSCC immunity and the IGF2BPs gene family is required. For example, the mechanisms of IGF2BPs modulation of immune pathways need to be studied beyond correlative analyses.

5. Conclusion

The current investigation revealed that the IGF2BPs gene family exhibited high expression levels in HNSCC, and could accurately predict this disease. The results also revealed that patients with HNSCC had a poor prognosis and had a significant expression of the IGF2BPs gene family. Furthermore, this research showed a direct link between the immunological microenvironment of HNSCC and the IGF2BPs gene family, and in vitro tests showed that IGF2BP2 knockdown could efficiently inhibit HNSCC cells from proliferating, and migrating, as well as limit their invasive capacities. According to these findings, the IGF2BPs gene family may be a useful biomarker and therapeutic target for HNSCC. To validate and facilitate the therapeutic application of these findings, additional research is needed.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

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CRediT authorship contribution statement

Hai Tang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Writing – original draft. **Jianjiang Zhao:** Funding acquisition, Supervision, Validation, Visualization, Writing – review & editing. **Jingpeng Liu:** Funding acquisition, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Nothing to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20659.

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