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ORIGINAL ARTICLE

Hypoxia-inducible factor-2α and its missense mutations: potential role in HCC diagnosis, progression, and prognosis and underlying mechanism

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Abstract

Objective This study aims to gain further the potential mechanisms of HIF-2α in tumor progression and tumorigenesis.

Methods Mined The Cancer Genome Atlas (TCGA) dataset. In total, 421 participants were enrolled in the TCGAHepatocellular Carcinoma (HCC) study, comprising 371 patients with cancer and 50 healthy controls. From the 371 tumor samples, three samples containing the missense mutation of the HIF-2α gene were compared with 368 wild-type samples to identify differentially expressed genes (DEGs).

Results After filtering, univariate Cox regression and multivariate Cox regression analyses showed that the differentially expressed genes (DEGs) progestagen-associated endometrial protein (PAEP) PNLIPRP2, MIR147B, and pregnancy zone protein (PZP) were significantly correlated with the survival times of patients with HCC. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 database to detect the functional annotation of these four DEGs as well as hub genes obtained from protein-protein interaction (PPI) network analysis using the STRING v10 database. Our analysis focused on the PAEP and PZP genes, whose protein expressions were downregulated in samples with HIF-2α missense mutation. The hub genes of PAEP and PZP were identified using PPI network analysis. Subsequent Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that PAEP and its hub genes were highly enriched in the TGF-β pathway, which is consistent with the analysis of PZP.

Conclusion Our study proved that the missense mutation of HIF-2α induces the upregulation of PAEP, which is positively related to the poor prognosis of patients with HCC, as it may upregulate the TGF-β pathway. In contrast, PZP downregulation showed the opposite phenomenon, as it may downregulate the TGF-β pathway.

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. With nearly 500,000 people dying from liver cancer each year, HCC is the third leading cause of cancer-related death [1]. This disease is frequently diagnosed at an advanced stage, when medical and surgical treatments are no longer available. HIF-2α was initially identified as the endothelial PAS domain protein (EPAS1), an endothelium-specific HIF-1α isoform. Thus, HIF-2α was considered to have a more specialized function than HIF-1α, and it also interacts with HIF-1α to execute various biological processes. HIF-2α has been shown to regulate enzymes in the glycolytic pathway in the absence of HIF-1α [2]. HIF-1α and HIF-2α also have common target genes, such as vascular endothelial growth factor A and glucose transporter 1, but the transcriptional activities of HIF-1α and HIF-2α differ in gene regulation [3]. Increased expression of HIF-2α has been observed in lung, breast, colorectal, and gastric cancers and has been associated with poor prognosis in many cases, except for liver cancer and acute myeloid leukemia [4]. Because of the observed opposing HIF-2α expression patterns and their correlation with HCC, special attention has been focused on the relationship between HIF-2α expression and HCC [5]. It has been found that the expression of HIF-2α can be lower or higher in HCC tissues, and HIF-2α expression is associated with a better or worse prognosis for HCC. It has been reported that there were HIF-2α mutations in patients with gangliocytic paraganglioma (GP). The mutated HIF-2α protein attenuated binding to the von Hippel–Lindau (VHL) protein, enhancing HIF-2α stabilization and activation, which consequently upregulate the HIF-2α downstream genes, contributing to the pathogenesis of cancer [6].

Methods

Gene expression and clinical data in The Cancer Genome Atlas database

Tissues from patients with HCC and adjacent normal tissues were obtained from RNA-seq gene expression version 2 (RNASeqV2) level 3 data (Illumina HiSeq platform) in The Cancer Genome Atlas (TCGA) database. All alteration data for missense mutations used in this study were obtained from the cBioPortal for Cancer Genomics (www.cBioPortal.org). Similar expression patterns of target genes were downloaded from the Gene Expression Profiling Interactive Analysis (GEPIA) database.

Functional annotation and protein-protein interaction network analysis

The online database STRING 10.5 (https://string-db.org/) was used to analyze protein interactions. Protein-protein interaction (PPI) network analysis was performed to identify the hub genes, pregnancy zone protein (PZP) and progestagen-associated endometrial protein (PAEP), which were enriched in the targeting pathway. Functional annotation of PAEP, PZP, and their hub genes was performed using the web tool of the updated version of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version v6.8.

Identification of NRF2-binding sites by in silico analysis

To identify the HIF-2α binding sites within the promoter regions of the putatively HIF-2α regulated genes, we used the transcription factor-binding site finding tool LASAGNA-Search 2.0, with cutoff values of $P \leq 0.01$. The search was limited to the 0–5 kb upstream promoter region relative to the transcription start site.

Gene set enrichment analysis

To further understand the association between the expression level of HIF-2α and biological processes, we performed gene set enrichment analysis (GSEA v2.2; http://www.broad.mit.edu/gsea/). All patients with HCC in the TCGA cohort were divided into two groups based on the median expression value of HIF-2α, and the respective HIF-2α expression level was used as the phenotype label. The thresholds for significance were determined by permutation analysis (1,000 permutations), and the false discovery rate (FDR) was calculated. A gene set was considered to be significantly enriched when the FDR score was < 0.25.

Results

Expression and biological functions of HIF-2α

Initially, HIF-2α was identified as the endothelial PAS domain protein, an endothelium-specific HIF-1α isoform. Therefore, it was considered to have a more specialized function than HIF-1α [2]. However, HIF-2α is also expressed in many other tissues, including the brain, heart, lung, kidney, liver, pancreas, and intestine, suggesting that it also has a widespread role in the response to hypoxia [7].

Recent data show that both HIF-1α and HIF-2α participate in hypoxia-dependent gene regulation through complex and sometimes antagonistic interactions in some cell types, such as kidney cancer cells [3]. HIF-1α preferentially induces the expression of genes encoding glycolytic enzymes, such as phosphofructokinase [8, 9] and lactate dehydrogenase A [10, 11]. In contrast, HIF-2α induces the expression of genes involved in tumor invasion, including matrix metalloproteinases (MMPs) 2 and 13 and the stem cell factor OCT–3/4 [12]. However, HIF-2α has also been shown to regulate enzymes in the glycolytic pathway in the absence of HIF-1α, and HIF-1α

is capable of activating some MMPs, suggesting that HIF-1α and HIF-2α play redundant roles. HIF-1α and HIF-2α also have common target genes, such as the vascular endothelial growth factor A (VEGFA) [3, 13, 14] and glucose transporter 1 [15], but the transcriptional activities of HIF-1α and HIF-2α are different in the regulation of these genes [12].

HIF-2α plays a vital role in embryonic development and is essential for catecholamine homeostasis [16] as well as neural [17] and hematopoietic development [18]. Knockout of HIF-2α in embryos causes developmental defects in several organs, including the retina, heart, lungs, liver, bone marrow, and muscle [19]. Recently, Lin et al. demonstrated that HIF-2α, but not HIF-1α, plays an important role in the embryonic development of hepatic outgrowth in zebrafish by directly controlling the expression of the leg1 gene [20].

The correlation between HIF-2α and cancer

Increased HIF-2α expression has been observed in lung cancer [21–24], breast cancer [25, 26], colorectal cancer [5], gastric cancer [27–29], pancreatic cancer [30, 31], liver cancer [32–35], prostate cancer [36], ovarian cancer [37], head and neck cancer [38, 39], ccRCC cancer [40], oral squamous cell carcinoma [41], and acute myeloid leukemia (AML) [4] and has been associated with different prognoses (Table 1). Special attention has been focused on the relationship between HIF-2α expression and HCC; opposite HIF-2α expression patterns and correlations have been observed in HCC [32–34]. It has been found that the expression of HIF-2α is lower or higher in HCC tissues and is associated with a better or worse prognosis in HCC [32–34].

A direct comparison of the functions of HIF-1α and HIF-2α in a KRAS-driven mouse model of lung tumorigenesis showed that HIF-1α deletion had a surprisingly little effect on tumor burden and progression, whereas the loss of HIF-2α increased tumor growth and progression [42]. The latter effect is correlated with HIF-2α driven expression of Scgb3a1, which encodes the putative tumor suppressor secret globin 3A1 [43]. Surprisingly, overexpression of a stabilized HIF-2α protein in the KRAS lung tumor mouse model also promotes tumor angiogenesis and invasion by increasing the expression of VEGFA and SNAIL [44].

The complex role of HIF-2α is also reflected at the cellular level. It has been reported that HIF-2α inhibits the growth of glioblastoma, SW480 colon cancer, liver cancer, and non-small cell lung cancer (NSCLC) cells but enhances the proliferation of other types of cancer cells, including gastric cancer, breast cancer, and Renalcellcarcinoma (RCC) cells [28, 45, 46]. Taken together, these findings prove that HIFs have dual and even opposite effects on tumor growth, which warrants careful consideration when using HIF inhibitors as cancer therapeutic agents.

The expression of HIF-2α is correlated with different cancer prognoses

To identify the relationship between the expression level of HIF-2α and various types of cancer, we mined data from TCGA. TCGA analysis revealed that the HIF-2α expression level in normal tissue was higher than that in tumor tissue in breast invasive carcinoma, prostate adenocarcinoma, ovarian serous cystadenocarcinoma, AML, lung adenocarcinoma, and lung squamous cell HCC (LUSC). We then determined whether HIF-2α expression level correlated with overall survival (OS) in cancer (Fig. 1a). We analyzed the data downloaded from TCGA using the R package version 3.4.3. The Kaplan-Meier curve revealed that patients with lower HIF-2α expression levels exhibited longer OS in HCC, which was consistent with the results for kidney renal clear cell HCC (KIRC) (Fig. 1b).

Identification of the expression of the different genes associated with HIF-2α in HCC

Our investigation revealed missense mutations in patients with HCC. To elucidate the mechanism of HIF-2α in HCC, we divided the 377 patients into two groups, which comprised three missense mutation samples or 368 wild-type samples, using the online analysis tool cBioPortal (www.cBioPortal.org) (Fig. 1c). Using the R-package "edgeR" for the identification of differentially expressed genes (DEGs) with RNA-seq expression profiles, we found 68 genes that were all downregulated (Fig. 1d). After filtering with $|\log FC| > 2$ and $P < 0.05$, we identified 40 DEGs between missense mutation and wild-type samples (Fig. 3). We also identified the DGEs between the 50 normal and 370 tumor tissues with the R-package "edgeR," the result of which revealed that there were 5,887 DEGs between the two groups. Using the Venny 2.1 tool, we obtained 19 overlapping downregulated genes by integrating the two datasets (Fig. 1e).

A risk score of four genes as an indicator for patients with HCC

Univariate Cox regression analyses were used to filter the identified 19 DEGs and selected genes with values of $P < 0.05$. The genes PAEP, PNLIPRP2 (pancreatic lipase-related protein 2), MIR147B (microRNA 147b), and PZP were significantly correlated with the survival time of patients with HCC. These four genes were selected for multivariate Cox regression analyses, which revealed that PAEP, PNLIPRP2, and MIR147B were highly expressed in the high-score group, and PZP was highly expressed in the low-score group (Fig. 2a). The coefficients of the four-gene signature are displayed in Table 2. Patients with high scores had significantly worse survival times than

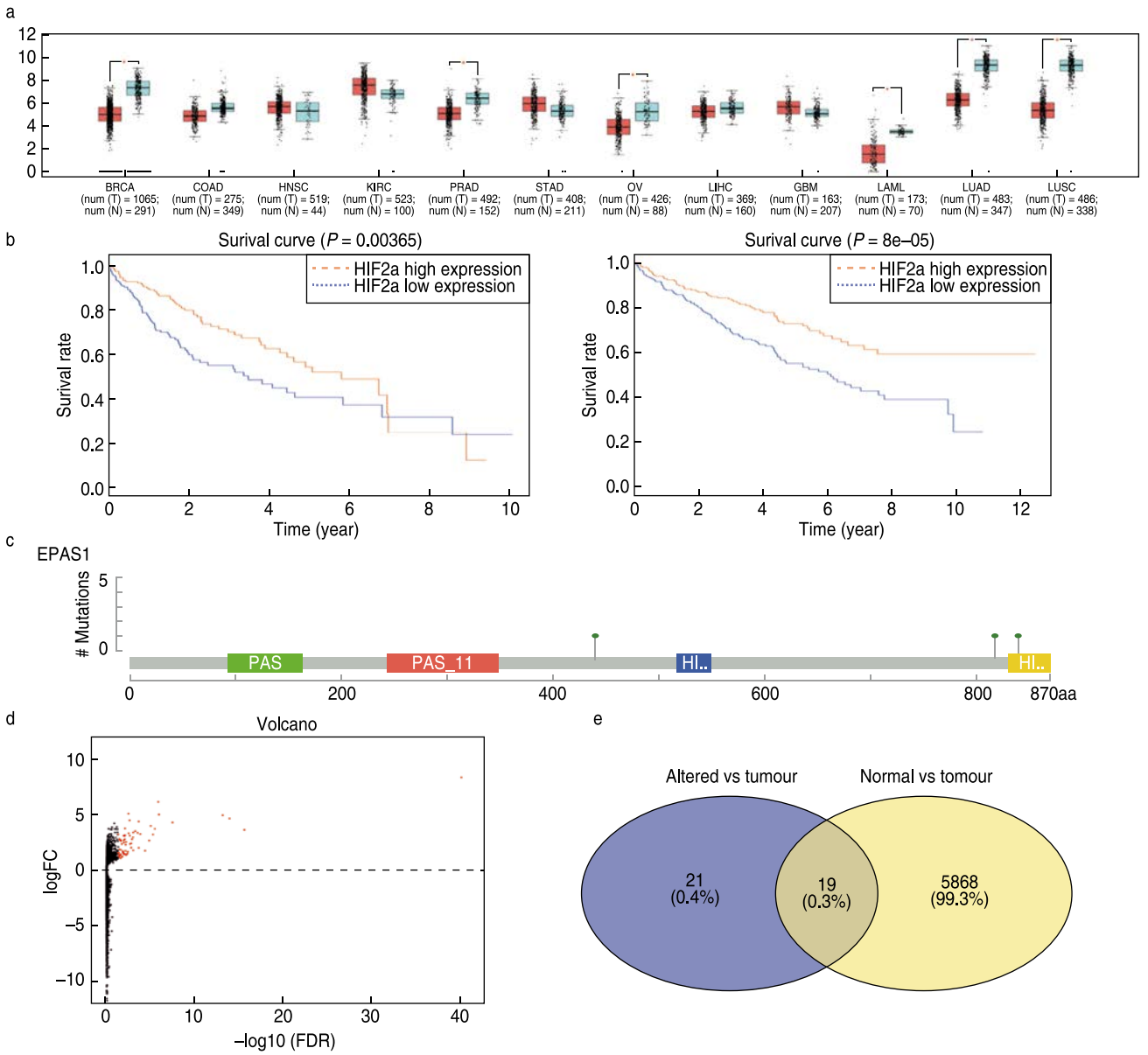

Fig. 1 The expression of HIF-2α correlated with different cancer prognosis and identification of the expression of the different genes associated with HIF-2α in HCC. (a) The gene expression of HIF-2α in normal tissue (green) compared with the tumor tissue (red); (b) KM overall survival curve in HCC and KIRC were according to the median value of the expression of HIF-2α; (c) cBioprotal-predicted mutation maps (lollipop plots) showing the mutation position of HIF-2α; (d) The upregulated genes in the missense mutation group exhibited in the volcano plot; (e) Nineteen overlapping upregulated genes were obtained by integrating the two datasets by utilizing the Venny 2.1 tool

those with low scores (Fig. 2b). Furthermore, these four genes exhibited superior capacity in predicting the 5-year survival rate, with an AUC value of 0.677 (Fig. 2c). Taken together, the score model of these four genes might serve as a significant predictive factor for prognosis in patients with HCC.

HIF-2α regulates the expression of putative oncogenes PAEP and PZP at the transcriptional level

To verify whether the transcription factor HIF-2α affects the gene expression of the four DEGs, we used LASAGNA-Search 2.0. The results revealed that HIF-2α

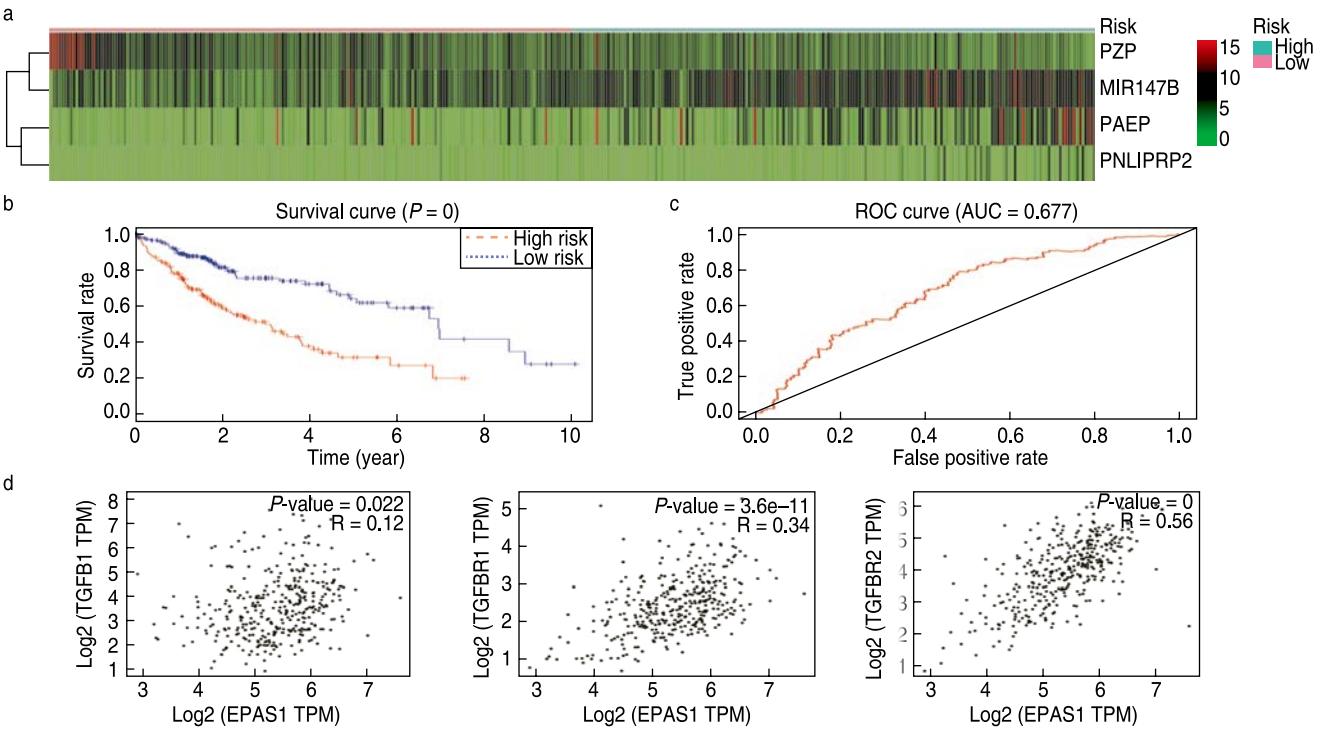

Fig. 2 Risk score of four genes as indicator in patients with HCC and GSEA plot showing that HIF-2α expression positively correlated with the TGF-β pathway and ECM Receptor Interaction in HCC. (a) Heat map showing the differential expression and risk of the four genes in HCC; (b) Kaplan-Meier survival curve: overall survival in patients with hepatocellular carcinoma according to the risk score; (c) The area under the curve was 0.677, demonstrating that the four-gene signature had high sensitivity and specificity for the classification of HCC patients from normal; (d) Expression levels of TF HIF-2α were positively correlated with the levels of TGFB1, TGFBR1, and TGFBR2 in TCGA dataset

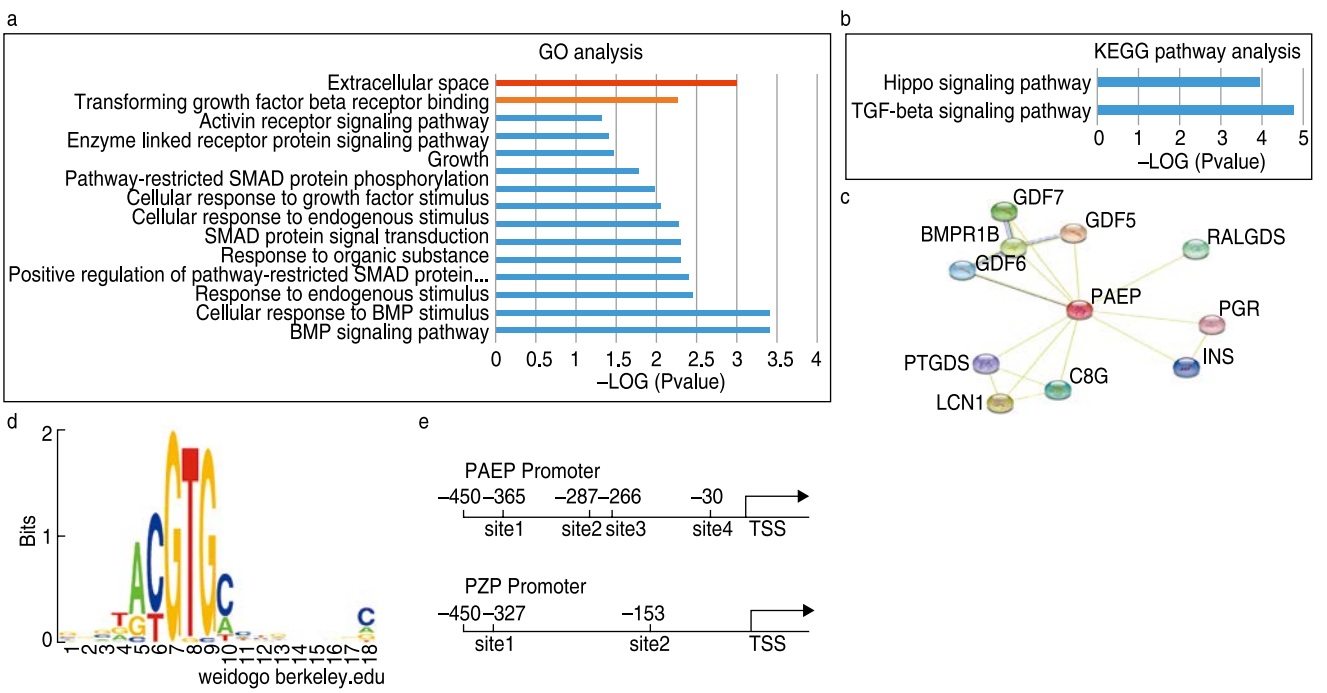

Fig. 3 HIF-2α binds to the ARE sequences of two putative genes identified in the four-gene signature and the KEGG, GO pathway prediction analysis, and PPI analysis of PAEP. (a–c). KEGG and GO pathway prediction analysis of PAEP, along with 10 hub genes. (d–e) Positions of in silico predicted HIF-2α binding sites (AREs) in the promoter regions of human PAEP and PZP

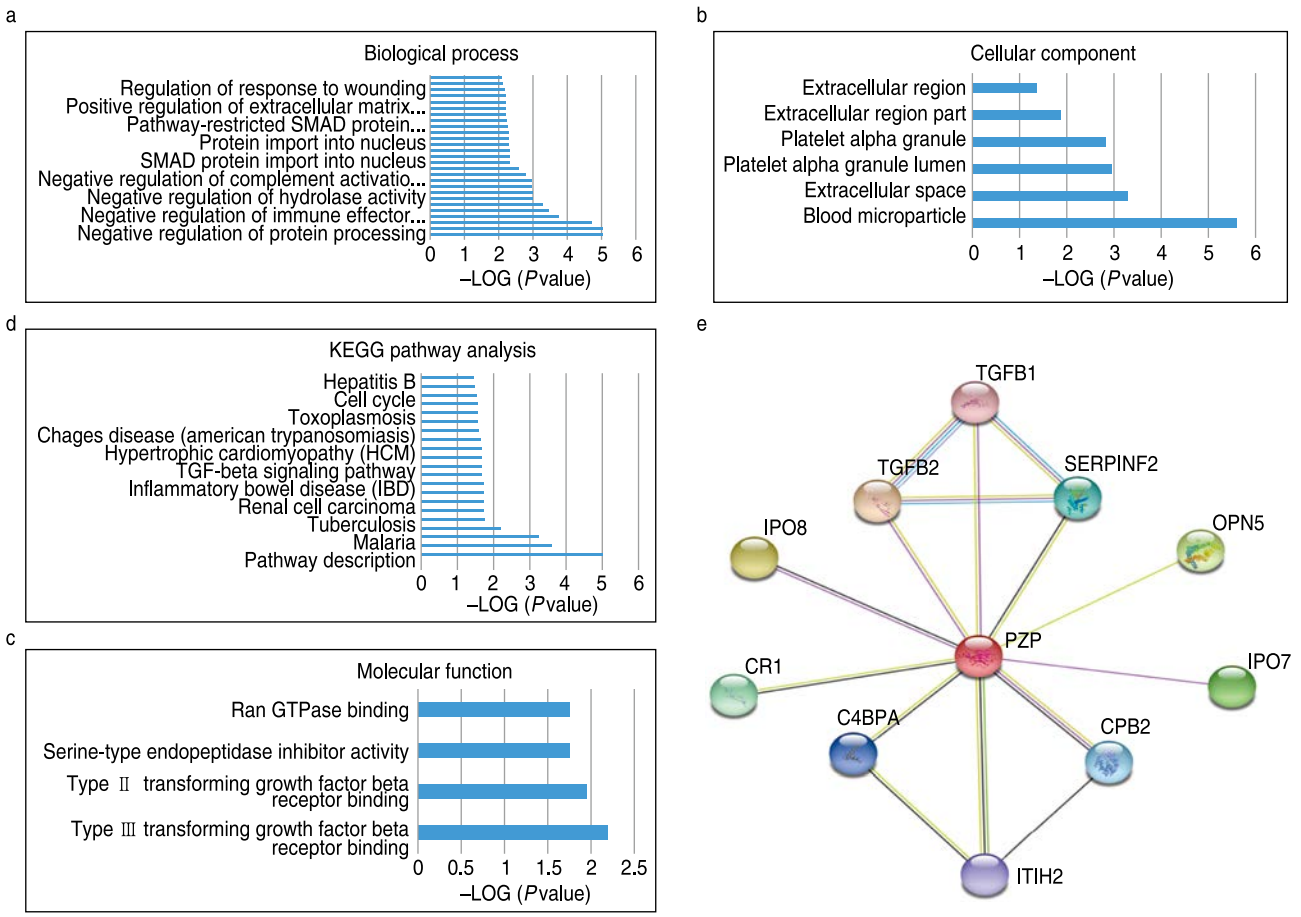

Fig. 4 KEGG, GO pathway prediction analysis, and PPI analysis of PZP. (a–c) GO Biological process, cellular component, and molecular function of PZP, along with its hub genes; (d) KEGG analysis for PZP and its hub genes; (e) Protein-protein interaction analysis for PZP and its hub genes

directly binds to the promoter regions of PAEP and PZP (Fig. 4d-e), which implies that HIF-2α affects the expression of PAEP and PZP by regulating their transcriptional levels. We then analyzed the functional annotation of PZP and the 10 hub genes from KEGG, GO pathway prediction analysis, and PPI analysis. The latter revealed that 10 hub genes were correlated with PZP (Fig. 3e). These genes are associated with 28 significant biological processes: negative regulation of cellular protein metabolic process, negative regulation of proteolysis, negative regulation of immune effector process, negative regulation of immune response, regulation of extracellular matrix organization, negative regulation of hydrolase activity, regulation of immunoglobulin-mediated immune response, negative regulation of macrophage cytokine production, negative regulation of complement activation, classical pathway, negative regulation of plasminogen activation, negative regulation of endopeptidase activity, SMAD protein import into the nucleus, negative regulation of fibrinolysis, platelet degranulation, protein import into the nucleus, protein targeting the nucleus, negative regulation of response to stimulus, pathway-restricted SMAD protein phosphorylation, regulation of catalytic activity, and regulation of response to stress (Fig. 3a). The TGF-β and Hippo signaling pathways were significantly enriched ($P < 0.05$) in the KEGG pathway analysis (Fig. 3d). By analyzing the PAEP using the same method, we found that PAEP, along with the 10 hub genes, highly enriched 13 significant biological processes: BMP signaling pathway, cellular response to BMP stimulus, response to endogenous stimulus, positive regulation of pathway-restricted SMAD protein phosphorylation, and response to organic substances (Fig. 4b–c), the TGF-β and Hippo signaling pathways were significantly enriched ($P < 0.01$) in the KEGG pathway analysis (Fig. 4a).

Discussion

At the transcriptional level, HIF-2α is a transcription factor that regulates the expression of other proteins. HIF-2α also interacts with proteins to execute different functions, which affects the progress and prognosis of various types of cancer. We have summarized that there are conflicting results in HCC xenograft tumor models.

He et al. found that downregulation of HIF-2α inhibits the growth of HCC tumors [47], and Sun et al. discovered that HIF-2α suppression does not affect HCC tumor growth, whereas HIF-2α overexpression reduces tumor growth [33]. GSEA revealed that HIF-2α was positively correlated with the TGF-β signaling pathway and ECM receptor interaction in patients with HCC. To further verify the mechanism of HIF-2α in HCC, we used the TCGA dataset to determine differences in the expression of DEGs between a missense mutation group and the corresponding wild-type group using cBioPortal (www.cBioPortal.org). We identified 19 overlapping upregulated genes by integrating the two datasets. Univariate and multivariate Cox regression analyses indicated that PAEP, PNLIPRP2, MIR147B, and PZP could predict the 5-year survival rate in patients with HCC. We used GEPIA to identify 100 genes with expression patterns similar to those of the four DEGs. After performing GO and KEGG pathway analyses with DAVID and STRING, we focused on biological processes, molecular functions, cellular components, and the pathways of PAEP and PZP in normal and tumor cells.

PAEP is a conflicting gene that affects the progression of various types of cancer. Overexpression of PAEP stimulates cell migration in human endometrial adenocarcinomas, and vascular endothelial growth factor mediates PAEP to facilitate neovascularization by increasing the migration and tube formation of human umbilical vein endothelial cells during embryogenesis and tumor development [48]. However, the overexpression of PAEP is correlated with differentiated epithelia and induces cell differentiation, which reduces the malignancy of cancer cells. Patients with high PAEP expression have worse survival times, which indicates that PAEP acts as a tumor gene in HCC. GO and KEGG pathway analyses, as well as PPI network analysis, revealed that PAEP interacts with 10 hub genes to increase the malignant characteristics of HCC cells and mediates the poor prognosis of patients with HCC by activating the TGF-β pathway. Taken together, the missense mutation of HIF2-α upregulated the expression of PAEP, which led to a worse prognosis in HCC.

The encoded protein of PZP is highly expressed in the late-pregnancy serum. It has been reported that the expression of PZP increases in the serum of individuals who later develop Alzheimer's disease (AD) [49]. In our study, we confirmed that the expression of PZP increased in HIF-2α missense-mutated samples compared with wild-type samples. Univariate Cox regression analyses revealed that PZP functions as a protective factor in HCC. Taken together, the missense mutation of HIF-2α upregulated PZP expression, which led to a better prognosis in HCC.

Taken together, we hypothesized that the missense mutation HIF-2α enhances the stabilization and activation of HIF-2α, which consequently upregulates the HIF-2α downstream genes PAEP and PZP, contributing to the conflicting roles of HIF-2α in HCC. HIF-2α is an endothelium-specific HIF-1α isoform with conflicting roles in various types of cancer. The mechanism of HIF-2α in tumor pathogenesis has not yet been elucidated, and further insight into the role of HIF-2α in cancer is required.

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Conflicts of interest

The authors declared that they have no conflicts of interest.

Author contributions

Not applicable.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethical approval

Not applicable.

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