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ORIGINAL ARTICLE

Basic Study

Prognostic value of *PEA3* subfamily gene expression in cholangiocarcinoma

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Abstract

BACKGROUND

Cholangiocarcinoma (CCA) is a lethal malignancy with limited treatment options and poor prognosis. The *PEA3* subfamily of E26 transformation specific genes: *ETV1*, *ETV4*, and *ETV5* are known to play significant roles in various cancers by influencing cell proliferation, invasion, and metastasis.

AIM

To analyze *PEA3* subfamily gene expression levels in CCA and their correlation with clinical parameters to determine their prognostic value for CCA.

METHODS

The expression levels of *PEA3* subfamily genes in pan-cancer and CCA data in the cancer genome atlas and genotype-tissue expression project databases were analyzed with R language software. Survival curve and receiver operating characteristic analyses were performed using the SurvMiner, Survival, and Procr language packages. The gene expression profiling interactive analysis 2.0 database was used to analyze the expression levels of *PEA3* subfamily genes in different subtypes and stages of CCA. Web Gestalt was used to perform the gene ontology/Kyoto encyclopedia of genes and genomes (GO/KEGG) analysis, and STRING database analysis was used to determine the genes and proteins related to *PEA3* subfamily genes.

RESULTS
ETV1, *ETV4*, and *ETV5* expression levels were significantly increased in CCA. There were significant differences in *ETV1*, *ETV4*, and *ETV5* expression levels among the different subtypes of CCA, and predictive analysis revealed that only high *ETV1* and *ETV4* expression levels were significantly associated with shorter overall survival in patients with CCA. GO/KEGG analysis revealed that *PEA3* subfamily genes were closely related to transcriptional misregulation in cancer. *In vitro* and *in vivo* experiments revealed that *PEA3* silencing inhibited the invasion and metastasis of CCA cells.

CONCLUSION
The expression level of *ETV4* may be a predictive biomarker of survival in patients with CCA.

Key Words: *PEA3* subfamily; Cholangiocarcinoma; The cancer genome atlas; *ETV4*; The prognosis; Prognostic biomarker

Core Tip: This study investigates the expression of the *PEA3* subfamily genes (*ETV1*, *ETV4*, *ETV5*) in cholangiocarcinoma (CCA) and their clinical relevance. Using data from cancer genome atlas and genotype-tissue expression project databases, we identified significantly elevated levels of *ETV1*, *ETV4* and *ETV5* in CCA. High expression of *ETV1* and *ETV4* was explicitly correlated with shorter overall survival in CCA patients. Functional assays demonstrated that silencing *PEA3* genes reduces invasion and metastasis in CCA cells *in vitro* and *in vivo*. These findings suggest that *ETV4* may be a valuable prognostic biomarker for survival of CCA patients.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a malignancy arising from bile duct epithelial cells. Although CCAs are rare, accounting for only 3% of gastrointestinal tumors, they are highly aggressive and have a poor prognosis[1,2]. CCAs can be divided into intrahepatic CCAs, perihilar CCAs, and distal CCAs according to anatomical location, with perihilar CCAs being the most common type, accounting for more than 60% of biliary tract tumors. Surgical resection is currently the treatment of choice and the treatment most likely to provide a cure. Most inoperable perihilar CCAs have a poor prognosis, with a median patient survival of less than one year[3].

Moreover, many CCAs are discovered at an advanced stage, which seriously affects patients' survival rates. Therefore, early screening for CCAs is essential for increasing survival. Although several previous studies have established omics profiles to reveal the underlying pathogenesis of CCA, there is still a lack of biomarkers for early diagnosis and predicting patient outcomes[4]. Therefore, the identification of biomarkers for early screening is essential.

The *PEA3* subfamily, a subset of the E26 transformation-specific (ETS) family, comprises the protein-coding genes *ETV1*, *ETV4*, and *ETV5*. These genes are overexpressed in various cancers and function as transcription factors that regulate cancer cells' proliferation, invasion, and metastasis. Abnormalities in or overexpression of *PEA3* subfamily genes have been linked to tumor onset and progression[5]. Research has shown that the expression levels of *PEA3* subfamily genes typically increase in liver cancer tissues, playing a crucial role in cancer cell invasion and metastasis[6]. Although *PEA3* subfamily genes are highly expressed in liver cancer, the study of their expression patterns in bile duct cancer is equally crucial for understanding the role of these genes in the underlying mechanisms of different cancer types and their potential therapeutic value, as this could provide new research directions for personalized medicine and precision treatment.

Unfortunately, research on CCA is limited because of insufficient relevant data. However, with advances in information technology and the establishment of the cancer genome atlas (TCGA) database, global bioinformatics-related research data can be shared, and data processing and analysis are more convenient. Therefore, research on the biological prognostic markers of CCA has become possible. In this study, the predictive value of the expression levels of different *PEA3* subfamily genes in CCA patients was evaluated by analyzing the relationships of these expression levels with clinical parameters.

MATERIALS AND METHODS

Bioinformatic databases

TCGA and GTEx Toil RNA-Seq data were downloaded from University of California Santa Cruz Xena (https://xenabrowser.net/data pages/). The expression data were first quantified in transcripts *per* kilobase million and then transformed [7]. There were 36 and 9 cancer and noncancer tissue samples in the CCA TCGA dataset. The R package "Limma" was used to compare the expression levels of *PEA3* subfamily genes between pancancer and CCA. Visualization of the data was performed using the ggplot2 R package.

Cell lines and mice

The CCA cell lines Rihoku bile duct epithelial (RBE) and human cholangiocarcinoma cells (HCCC)-9810 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were all maintained in F-12K medium (Cytiva, China) supplemented with 100 mL/L fetal bovine serum (Cytiva, China), penicillin (100 μg/mL), and streptomycin (100 μg/mL). All the cells were incubated in a humidified incubator with 50 mL/L carbon dioxide (CO_2) air at 37 °C. The medium was changed every 2 d, and the cells were passaged when they reached 70%-90% confluence.

In this experiment, 4-6 weeks BALB/c male nude mice (Shanghai BK Company, China) weighing 20-25 g were used, and all mice were raised in the specific pathogen-free animal room of Zhejiang University of Traditional Chinese Medicine. Zhejiang University of Traditional Chinese Medicine provided the feed and bedding used in the experiment. The feeding process followed the guidelines of the Animal Protection and Use Committee of Zhejiang University of Traditional Chinese Medicine. This study was approved by the Animal Ethics Committee of Zhejiang University of Traditional Chinese Medicine (ethical batch number: IACUC-20230410-21).

The number of HCCC-9810 cells transfected with the NC-vector or shRNA-*PEA3* was adjusted to 5.0×10^6 cells *per* milliliter in phosphate buffer saline (PBS). The cell suspension (100 μL) was then injected into the left armpit of BALB/c mice, and mice were regularly evaluated for tumor formation. Approximately six weeks after subcutaneous injection, the BALB/c mice were killed by cervical dislocation, and the size and weight of the subcutaneous tumors were measured.

Bioinformatics analysis

Gene expression levels were validated based on information in the online database Oncomine[8]. The threshold parameters were $P < 0.001$, fold change = 2, and top gene rank = 10%. The gene expression profiling interactive analysis 2.0 database was used to analyze the expression levels of *PEA3* subfamily genes in different CCA tumor subtypes[9]. In this study, we used WebGestalt to perform gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses[10], and we analyzed the functional protein association network using the STRING database[11].

Prognostic prediction

The level-3 expression, clinicopathological, and prognostic information from the TCGA-cholesterol project were downloaded from the TCGA database. The R packages Survminer (version 0.4.6) and Survival were used for survival analysis[12]. Receiver operating characteristic (ROC) curve analysis was performed using pROC (v1.10.0).

Functional enrichment

Functional enrichment analysis was performed using the Web Gestalt program. The screening criteria for GO and KEGG analyses were $|\log \text{fold change}| > 0.45$, adjusted P consistency < 0.05, and $|\log \text{fold change}| > 0.2$, revised $P < 0.05$, respectively.

Network interaction analysis data related to the three genes in the *PEA3* subfamily were obtained from the STRING online database. *Homo sapiens ETV1*, *ETV4*, and *ETV5* was used as the analysis object, with the minimum interaction requirement score set to 0.400. Text mining was performed, and the database and experiments were used as active interaction sources.

Immunohistochemical

Paraffin sections (5 μm thick) were dewaxed in xylene I and II every 10 min, gradually dehydrated in 100%, 95%, 90%, 80%, and 70% absolute ethanol solutions every 5 min, and then boiled in distilled water for 15 min. After blocking the sections with 100 mL/L serum-containing blocking solution at room temperature for 1 hour, anti-*PEA3* (1: 1000; ab189826; Abcam; United States) was added, and sections were incubated with the anti-*PEA3* overnight at 4 °C. Sections were then incubated with secondary antibodies at room temperature for 30 min. The horseradish peroxidase-labeled antibodies were developed with diaminobenzidine. After counterstaining with hematoxylin, the sections were dehydrated and sealed.

Cell migration and invasion assay

In the top chamber of the Transwell system (8-μm pore), 200 μL of a suspension of CCA cells (5×10^4 cells) was seeded and incubated for 24 hours at 37 °C in the air with 50 mL/L CO_2. The bottom of the Transwell chamber was filled with F-12K media supplemented with 200 mL/L fetal bovine serum. Cotton swabs were used to remove nonmigrating cells carefully. The cells were fixed in methanol for 5 min before being stained for 30 min with crystal violet (Beyotime, China). The chambers were gently rinsed with deionized water to remove floating color, and the number of invading cells was counted using a microscope.

Cell colony-forming assay

CCA cells were plated at a low density (5000 cells/100-mm plate) and incubated for 10 d at 37 °C in 50 mL/L CO_2. The medium was removed, and the cells were washed with PBS before fixation with 40 g/L paraformaldehyde at 37 °C for 10 min. After incubation with 5 mL/L crystal violet for 15 min at 37 °C, the wells were washed 3 times with PBS, air dried, and examined for colony morphology. Statistical analysis was performed, and a histogram was constructed after the data from three separate repetitions were analyzed.

Western blot analysis

The suspension of CCA cells was lysed for 30 min on ice in radio immunoprecipitation assay buffer containing 1 mL/L phenylmethanesulfonyl fluoride (Solarbio, China) and then centrifuged for 10 min (12000 × g, 4 °C). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime, China) was used to resolve total protein, and samples were electro transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, United States) in transfer buffer. The PVDF membrane was blocked with 5 mL/L skim milk at 37 °C for 2 hours, and the membranes were treated with anti-*PEA3* (1: 1000; ab189826; Abcam; United States) at 4 °C overnight. The membranes were subsequently incubated with Horseradish Peroxidase-conjugated goat anti-rabbit IgG (Servicebio, China) at 37 °C for 2 hours. β-actin was used as a loading control for Western blots. A sensitive enhanced chemiluminescence kit (Meilunbio, China) was used to measure the immunoreactivity of the bands, which were visualized with the General Electric AI800 gel imaging system.

shRNA transfection assay

CCA cells were transfected with standard control shRNA or *PEA3*-shRNA from Shandong Weizhen Co., Ltd (Shandong province, China) using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Invitrogen, United States) according to the manufacturer's instructions. Transfection efficiency was examined by Western blot.

Statistical analysis

R version 4.0.3 from the R Studio software package and statistical product and service solutions (SPSS) 19.0 software (SPSS, Inc., Chicago, IL, United States) were used for statistical analysis. The R packages Survminerand Survival were used for survival analysis. ROC analysis was performed using pROC. The differences between shRNA-*PEA3* and control cells *in vivo* and *in vitro* were evaluated using independent sample *t*-tests. $P < 0.05$ was considered statistically significant in all comparisons.

RESULTS

The expression of the PEA3 subfamily in patients with pan-cancer and CCA from databases

First, we compared the expression levels of *PEA3* subfamily genes and found that these genes were overexpressed in many cancers, indicating that *PEA3* subfamily genes primarily depend on cancer type (Figure 1A-C). Second, we confirmed that the Oncomine database showed the same results (Figure 1D). The results also revealed that *ETV1*, *ETV4*, and *ETV5* were highly expressed in CCA (Figure 2).

The relationship between PEA3 subfamily expression level and clinicopathological parameters of CCA patients from databases

Compared with those in healthy controls, the expression levels of *ETV1*, *ETV4*, and *ETV5* in patients with different tumor subtypes were significantly greater (all $P < 0.05$) (Figure 3). When the expression levels of *PEA3* subfamily genes according to the stage of CCA were compared, there was no significant difference in the expression levels of *ETV1*, *ETV4*, and *ETV5* in CCAs in different anatomical locations ($P = 0.727$) (Figure 4).

High ETV4 expression is associated with poor prognosis of CCA patients from databases

Cox regression revealed no significant difference in the survival time distribution between the high *ETV1* and *ETV5* expression group and the low *ETV1* and *ETV5* expression group ($P > 0.05$) (Figure 5A and C). However, the survival time of patients in the high *ETV4* expression group was significantly shorter than that of patients in the low *ETV4* expression group ($P = 0.04$) (Figure 5B). ROC curve analysis revealed that *ETV1*, *ETV4*, and *ETV5* expression levels accurately predicted tumors (Area under the curve = 0.907, 0.954, 0.978) (Figure 5D).

GO/KEGG analysis of the PEA3 subfamily genes and their co-expressed genes in TCGA

Co-expression analysis of data from the STRING database revealed that *PEA3* subfamily genes were co-expressed with *JUN*, *MAPK14*, *MAPKAPK2*, *NCOA3*, and *STK11* (Figure 6).

Biological process terms included: (1) GO: 0008152 (metabolic process); (2) GO: 0032502 (developmental process); (3) GO: 0065007 (biological regulation); (4) GO: 0016043 (cellular component organization); (5) GO: 0032501 (multicellular organismal process); (6) GO: 0050896 (response to stimulus); (7) GO: 0000003 (reproduction); (8) GO: 0007154 (cell communication); (9) GO: 0008283 (cell proliferation); (10) GO: 0051179 (localization); and (11) GO: 0051704 (multiorganism process) (Figure 7A).

The cellular component terms included: (1) GO: 0005634 (nucleus); (2) GO: 0031974 (membrane-enclosed lumen); and (3) GO: 0016020 (membrane) (Figure 7B). The molecular function terms included: (1) GO: 0003676 (nucleic acid binding);

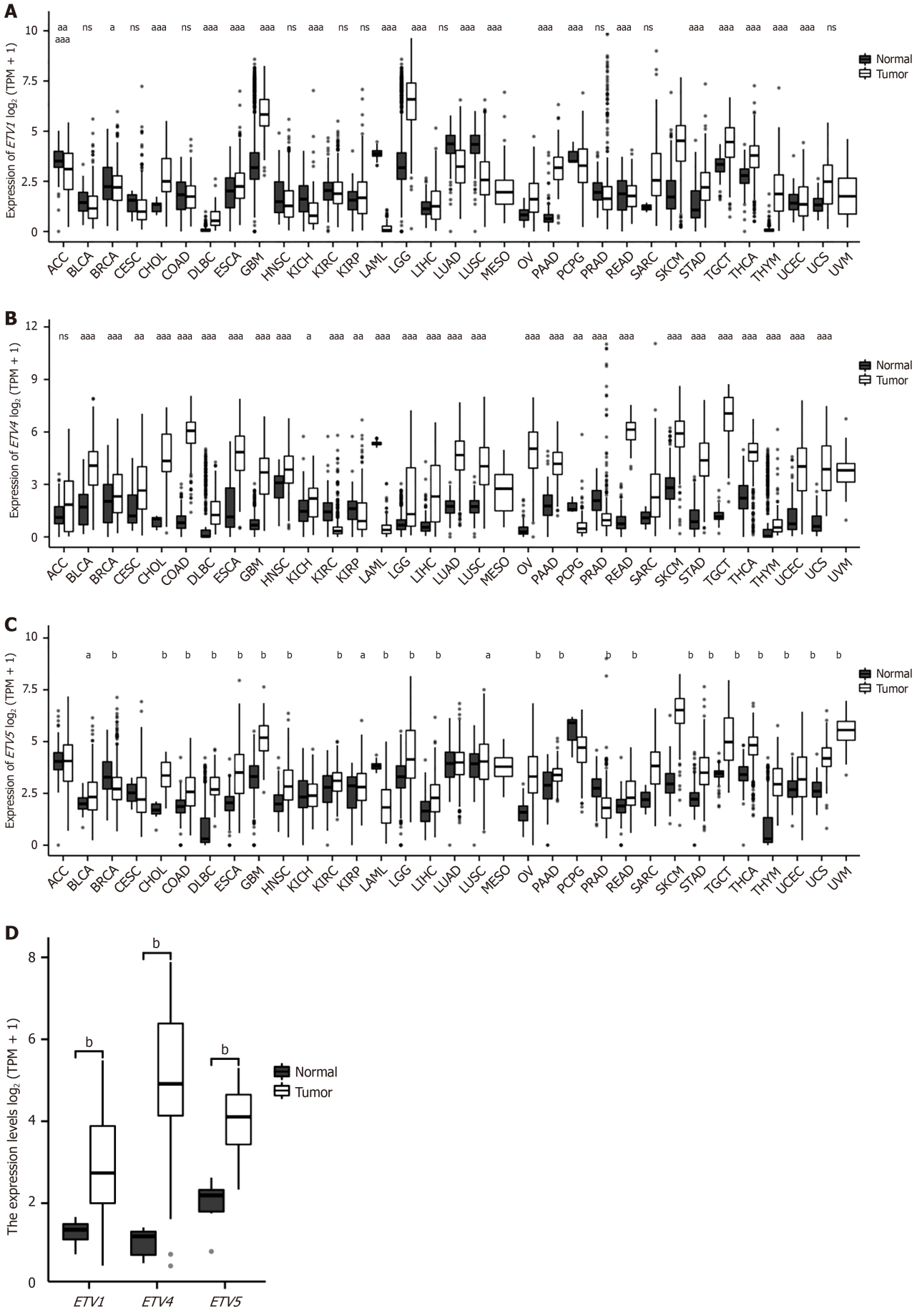


Figure 1 The expression of the *PEA3* subfamily in patients with pan-cancer and cholangiocarcinoma. A: The expression of *ETV1* in pan-cancer;

B: The expression of *ETV4* in pan-cancer; C: The expression of *ETV5* in pan-cancer; D: The boxplot of *PEA3* subfamily expression in tumor and normal tissues. [a]*P* < 0.05. [b]*P* < 0.01. TPM: Transcripts *per* million; ACC: Adrenocortical carcinoma; BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma multiforme; HNSC: Head and neck squamous cell carcinoma; KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute myeloid leukemia; LGG: Brain lower grade glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin cutaneous melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular germ cell tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine corpus endometrial carcinoma; UCS: Uterine carcinosarcoma; UVM: Uveal melanoma.

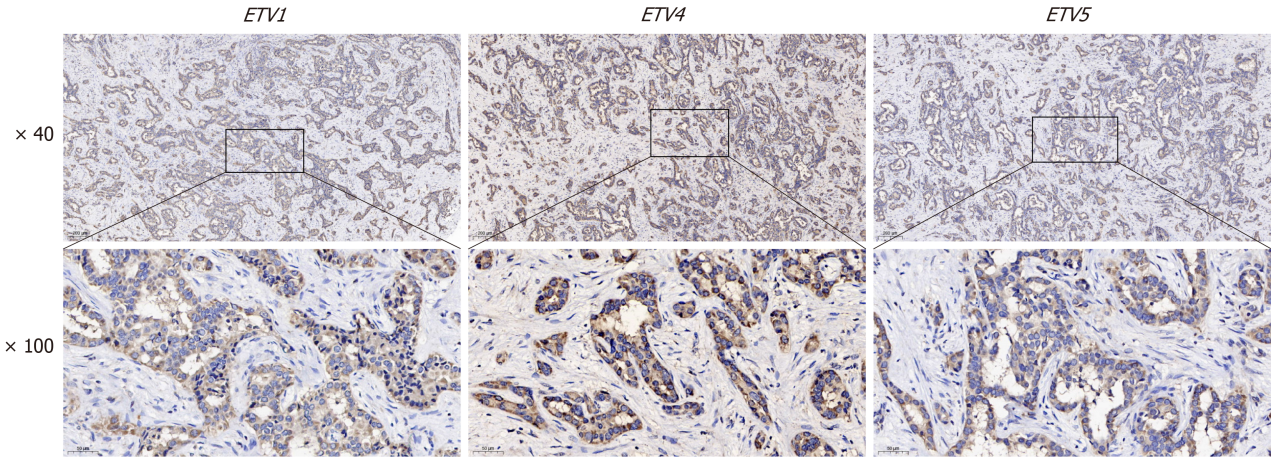


Figure 2 *PEA3* subfamily expression in cholangiocarcinoma. A: The expression of *ETV1* in different subtypes of cholangiocarcinoma (CCA) was significantly higher than that in normal group; B: The expression of *ETV4* in different subtypes of CCA was significantly higher than that in normal group; C: The expression of *ETV5* in different subtypes of CCA was significantly higher than that in normal group.

and (2) GO: 0005515 (protein binding) (Figure 7C).

KEGG analysis revealed that the *PEA3* subfamily genes are related to hsa05202: Transcriptional misregulation in cancer.

In vitro experiments: Silencing PEA3 subfamily genes inhibits the invasion and metastasis of CCA cells

PEA3 subfamily gene expression levels decreased in RBE and HCCC-9810 cells following transfection with shRNA-*PEA3* (Figure 8A and B).

After being transfected with shRNA-*PEA3*, the capacity of RBE and HCCC-9810 cells to proliferate and invade dramatically decreased in invasion and migration assays (Figure 8C-E) and colony formation experiments (Figure 8F and G).

In vivo experiments: Silencing PEA3 subfamily genes inhibits the invasion and metastasis of CCA cells

To verify that inhibition of PEA3 subfamily genes can inhibit the proliferation of CCA cells, nude mice were inoculated with shRNA-*PEA3*-transfected RBE cells or un transfected RBE cells and rate of subcutaneous tumor volume increase was significantly lower, and the tumor weight was lower in the shRNA-*PEA3* group than the control group (Figure 9).

DISCUSSION

The ETS family represents one of the most prominent families of signal-dependent transcription factors[13]. ETS transcription factors are divided into several subfamilies on the basis of their degree of amino acid conservation and subgroup-specific amino acid sequence in the ETS domain[4]. As members of the *PEA3* subfamily, *ETV1*, *ETV4*, and *ETV5* share an ETS domain and an N-terminal domain of the *PEA3*-type ETS transcription factor. *ETV1*, *ETV4*, and *ETV5* participate in tumorigenesis and development by regulating various biological processes (including cell proliferation, migration, apoptosis, epithelial-mesenchymal transition, and maintenance of the cancer stem cell phenotype)[4,14]. However, relatively few studies have investigated the expression of *PEA3* subfamily genes in CCA. In this study, data from various bioinformatic databases were used to evaluate the correlation between *PEA3* subfamily gene expression levels and CCA clinical parameters and to explore the prognostic value of *PEA3* subfamily gene expression levels in CCA.

The results of the database analyses performed in this study revealed that the expression levels of the *PEA3* subfamily genes mainly depended on cancer type, with high expression levels in most cancers. Previous studies revealed high *ETV1*, *ETV4*, and *ETV5* expression levels in many cancers. For example, *ETV1* is highly expressed in prostate cancer[15] and gastrointestinal stromal tumors[16]. *ETV4* is frequently activated in gastric[17], lung[18], hepatocellular[19], and colore-

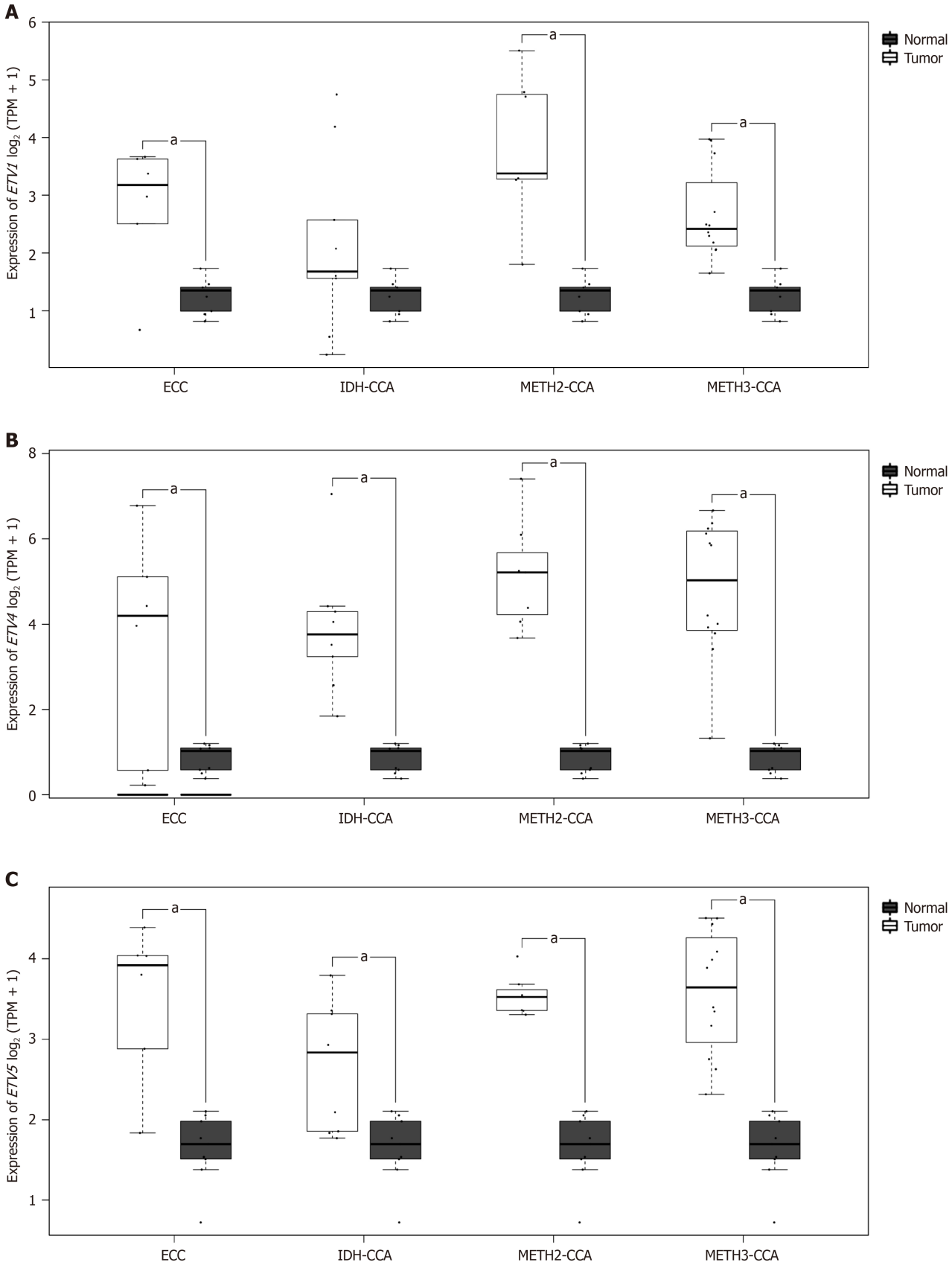


Figure 3 Box plots of normal and tumor differential expression of *PEA3* subfamily members in different subtypes of cholangiocarcinoma.
[a]*P* < 0.05. TPM: Transcripts *per* million; ECC: Extrahepatic cholangiocarcinoma; IDH-CCA: Isocitrate dehydrogenase cholangiocarcinoma; METH2-CCA: Methylation cluster 2 cholangiocarcinoma; METH3-CCA: Methylation cluster 3 cholangiocarcinoma.

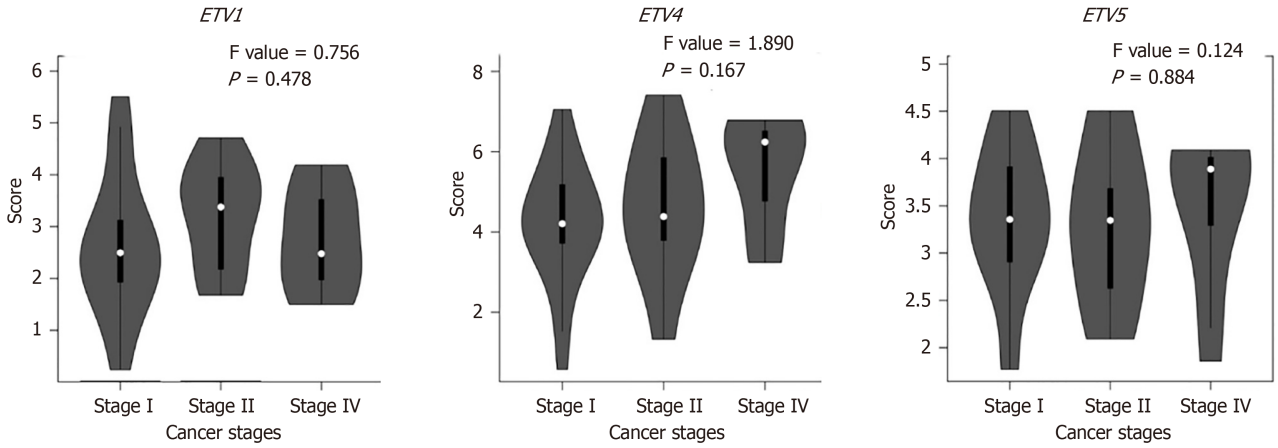


Figure 4 Relationship between expression of distinct *PEA3* subfamily family members and individual cancer stages of cholangiocarcinoma patients.

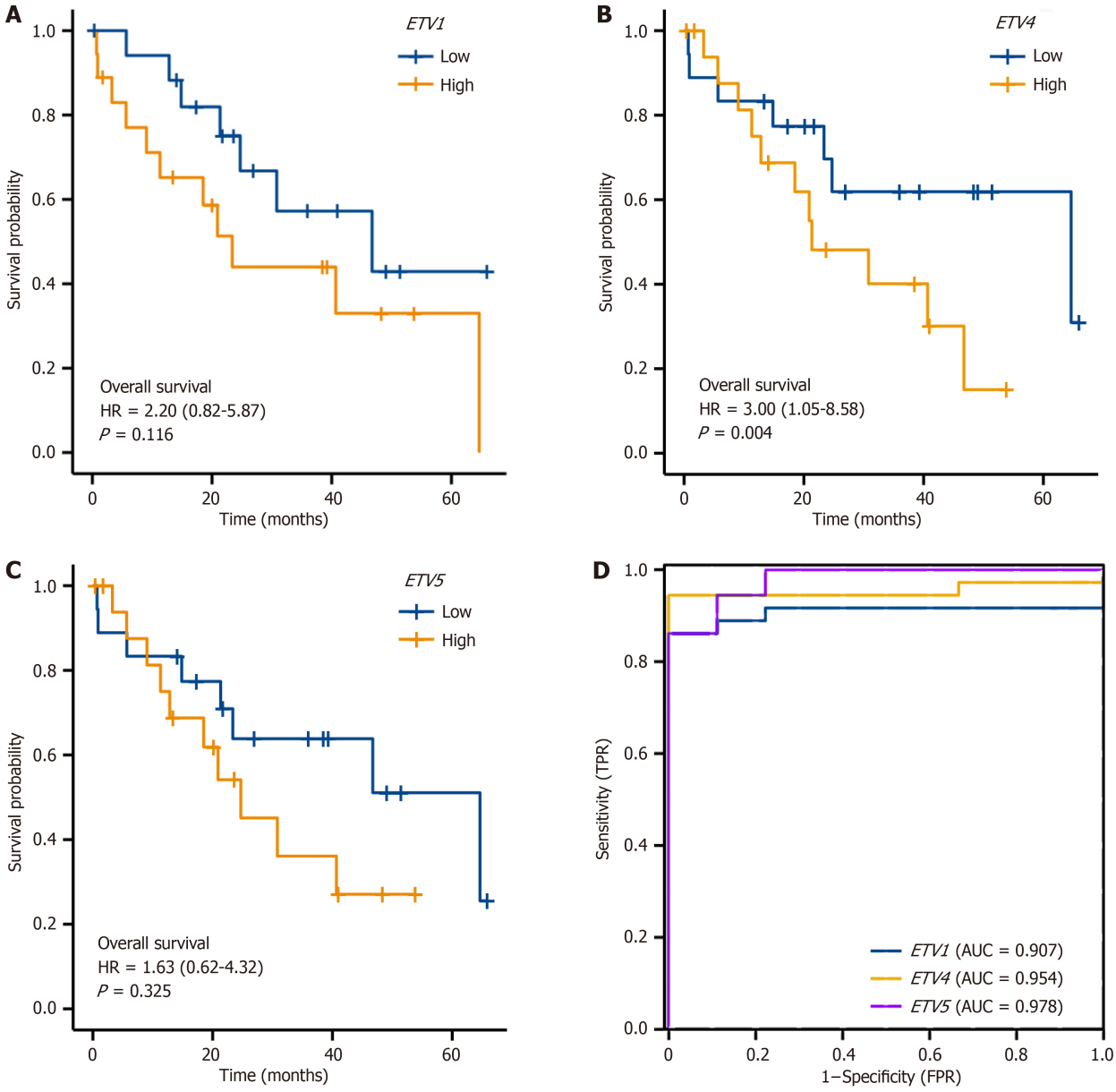


Figure 5 Prognostic value of expression of distinct *PEA3* subfamily members in cholangiocarcinoma patients. A: *ETV1* expression showed no

correlation with prognosis in cholangiocarcinoma (CCA) patients B: Higher expressions of *ETV4* were significantly associated with shorter overall survival of CCA patients; C: *ETV5* expression showed no correlation with prognosis in CCA patients; D: *ETV1*, *ETV4*, and *ETV5* has high accuracy in predicting tumors (area under curve = 0.907, 0.954, 0.978). HR: Hazard ratio; FPR: False positive rate; TPR: True positive rate.

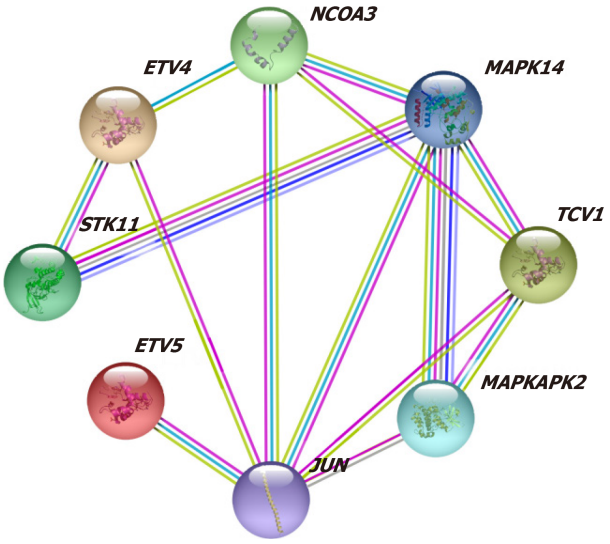


Figure 6 The network of splice isoforms or post-translational modifications for *PEA3* subfamily function.

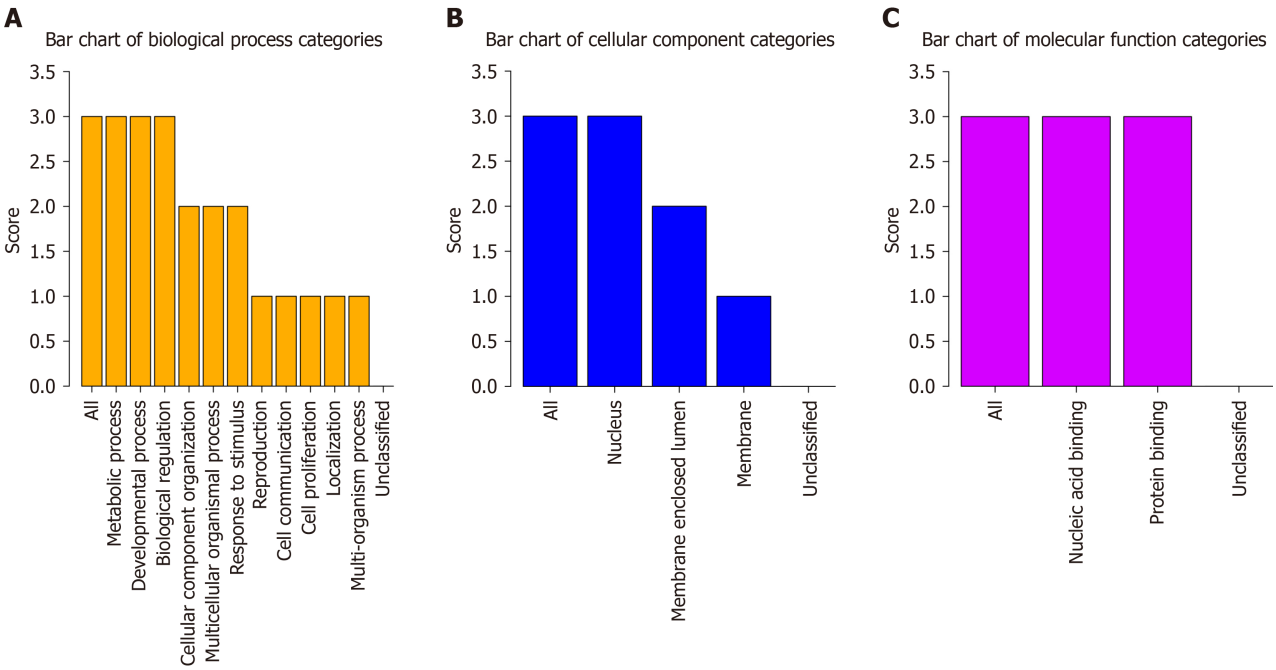
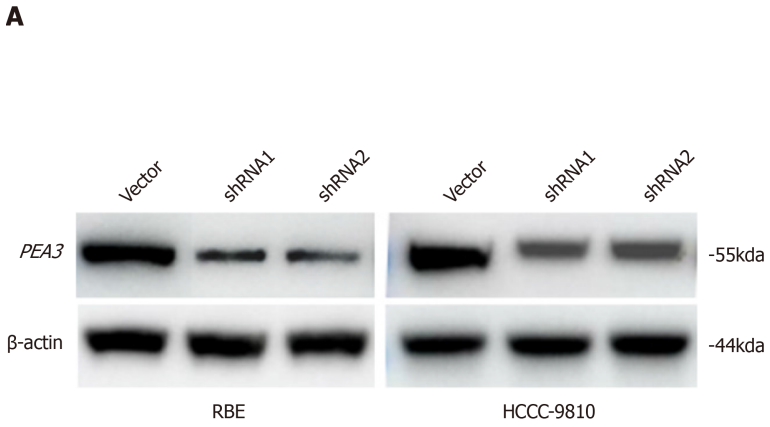


Figure 7 Gene-annotation enrichment analysis of *PEA3* subfamily for gene ontology. A: Biological process; B: Cellular component; C: Molecular function.

ctal[20] cancers. *ETV5* is related to endometrial[21] and ovarian cancer[22] progression. This study revealed that *ETV1*, *ETV4*, and *ETV5* was highly expressed in CCA patients compared with healthy individuals. The expression levels of *ETV1*, *ETV4*, and *ETV5* were significantly greater in different CCA subtypes but were not significantly different across CCA stages. This finding may be related to further activating molecular mechanisms involved in the *PEA3* subfamily-related pathways. Upstream regulatory signals from the mitochondrial gene-activated protein kinase and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways are important causes of *ETV1*, *ETV4*, and *ETV5* overexpression in different tumor types[23,24].

While ROC curve analysis revealed that *ETV1*, *ETV4*, and *ETV5* expression levels had value in predicting CCA, the predictive analysis revealed that only high *ETV4* expression was significantly correlated with shorter overall survival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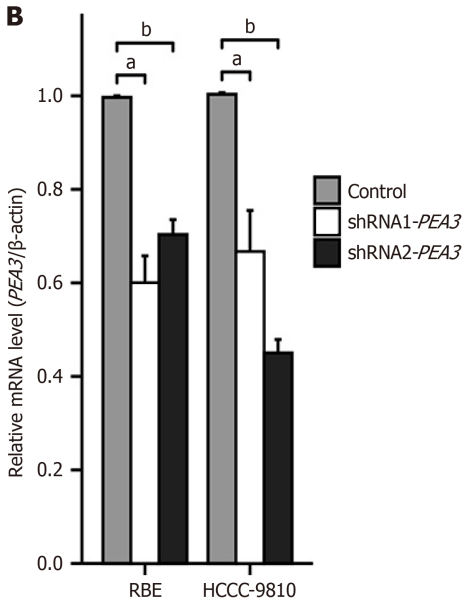
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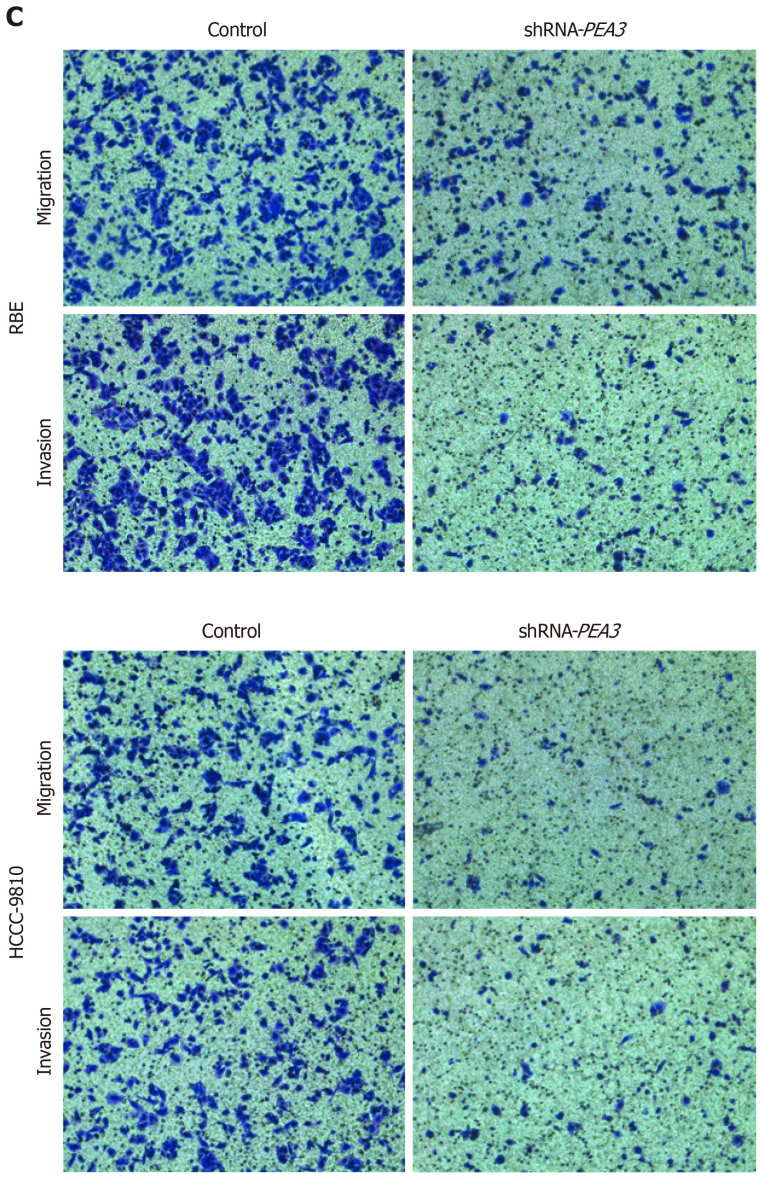
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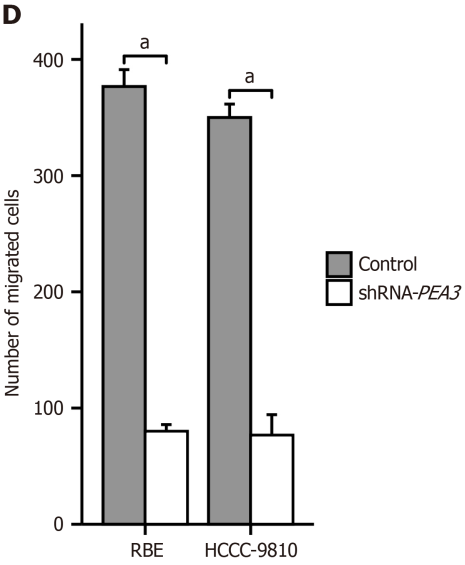
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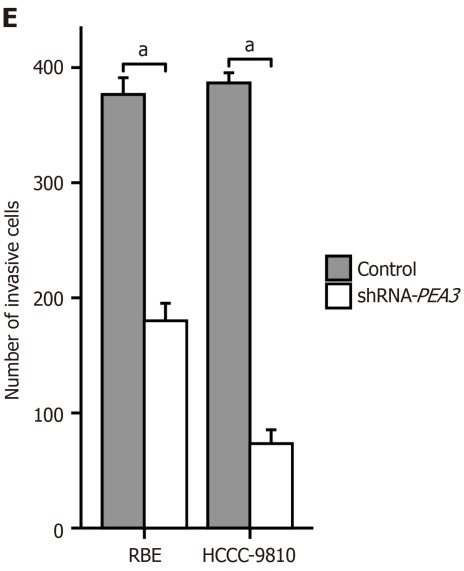
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HCCC-9810

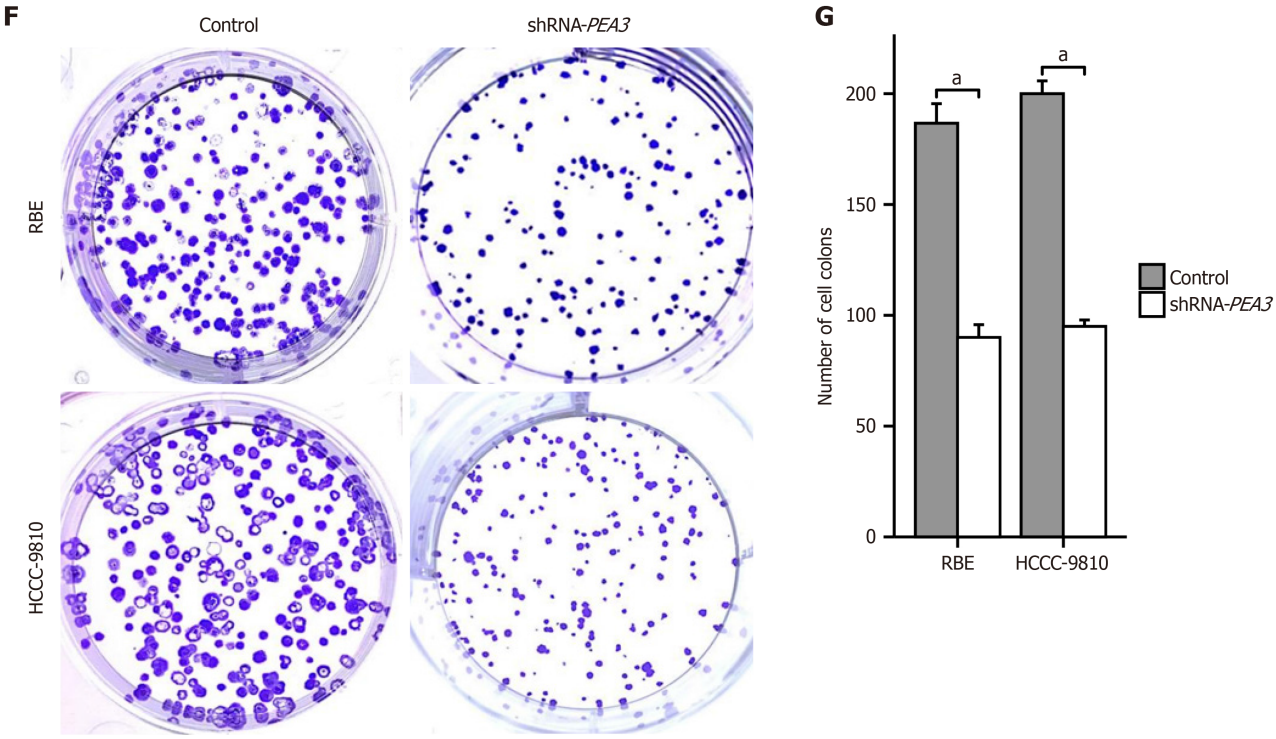

Figure 8 Inhibition of cholangiocarcinoma cell function after transfection with shRNA-*PEA3* in vitro. A: *PEA3* expression was considerably decreased in Rihoku bile duct epithelial (RBE) and human cholangiocarcinoma cells-9810 followed transfection with shRNA-*PEA3*; B: Statistics of the relative mRNA level; C: Transwell analysis showed the migration and invasion ability of RBE cells transfected with shRNA-*PEA3*; D: Statistics of the number of migrated cells; E: Statistics of the number of invading cells; F: Colony formation assay showing the proliferative capacity of RBE cells after transfection with shRNA-*PEA3*; G: Statistics of the number of colon cells. $^{a}P < 0.05$. $^{b}P < 0.01$. RBE: Rihoku bile duct epithelial; HCCC-9810: Human cholangiocarcinoma cells-9810.

(OS) in patients with CCA. Prior studies in various cancers have demonstrated that the expression levels of *PEA3* subfamily genes are positively correlated with tumor depth, lymph node metastasis, and recurrence, leading to shorter OS and disease-free survival, making high expression levels of *PEA3* subfamily genes independent of adverse prognostic factors[4,17-22]. Specifically, a high *ETV4* expression level can predict colorectal cancer progression and metastasis[21], whereas a high *ETV5* expression level is an independent predictor of liver cancer prognosis[19,25]. In CCA, the *ETV4* expression level has prognostic value in other databases, *ETV4* is also a potential intermediate in β-estradiol activation of CCA and a target of the estrogen antagonist tamoxifen-mediated tumor inhibition observed in animal models[26].

In our study, protein interaction network analysis revealed that *PEA3* subfamily genes were co-expressed with *JUN*, *MAPK14*, *MAPKAPK2*, *NCOA3*, and *STK11*. GO/KEGG analysis revealed that the *PEA3* subfamily genes were closely related to transcriptional mis-regulation in cancer. The N-terminus of the *PEA3* transcription factor in the *PEA3* subfamily has a conserved mitogen-activated protein kinase (MAPK) phosphorylation site. This site can increase transactivation through MAPK pathway activation and the inhibition of DNA binding, which results in the occurrence and development of tumors[14]. Among the posttranslational modifications of *PEA3* subfamily genes are phosphorylation and acetylation for *ETV1* and *ETV4* and phosphorylation and ubiquitination for *ETV5*[5].

The molecular function of *PEA3* subfamily genes is related mainly to nucleic acid and protein binding. *PEA3* subfamily genes are closely related to biological processes such as metabolism, neural development, reproductive capacity, cell proliferation, motor coordination, axon guidance, hormone regulation, and tumorigenesis. Owing to the different expression levels of *ETV1*, *ETV4* and *ETV5* in various tissues and organs, the functions of these transcription factors differ. For example, *ETV4* and *ETV5* play crucial roles in average axon growth and development[27]; *ETV4* and *ETV5* can also promote oocyte maturation and ovulation by upregulating cyclooxygenase 2[28]. Additionally, *ETV5* can promote the self-renewal of female germline stem cells, which are essential for normal spermatogenesis[29]. This study further confirmed through *in vivo* and *in vitro* experiments that inhibiting *PEA3* subfamily genes can suppress the invasion and metastasis of CCA cells. These results support previous research findings that *PEA3* subfamily genes, as transcription factors, play a crucial role in tumor cell proliferation, invasion, and metastasis[5]. The specific underlying mechanism may involve multiple signaling pathways in the tumor microenvironment, such as the activation of the MAPK/extracellular regulated protein kinases signaling pathway or the PI3K/Akt signaling pathway[30,31].

There are limitations to this study. First, further studies are needed to clarify the specific mechanism by which the silencing of *PEA3* subfamily genes inhibits the invasion and metastasis of CCA cells. Second, previous studies have shown that targeting *PEA3*-related genes or pathways or directly targeting *PEA3* subfamily genes can overcome drug resistance and increase the efficacy of cancer therapy[32], but this study did not evaluate the potential diagnostic and therapeutic value of the expression levels of *PEA3* subfamily genes for CCA; thus, further studies should explore whether the expression of *PEA3* subfamily genes could serve as a diagnostic marker or therapeutic target for CCA.

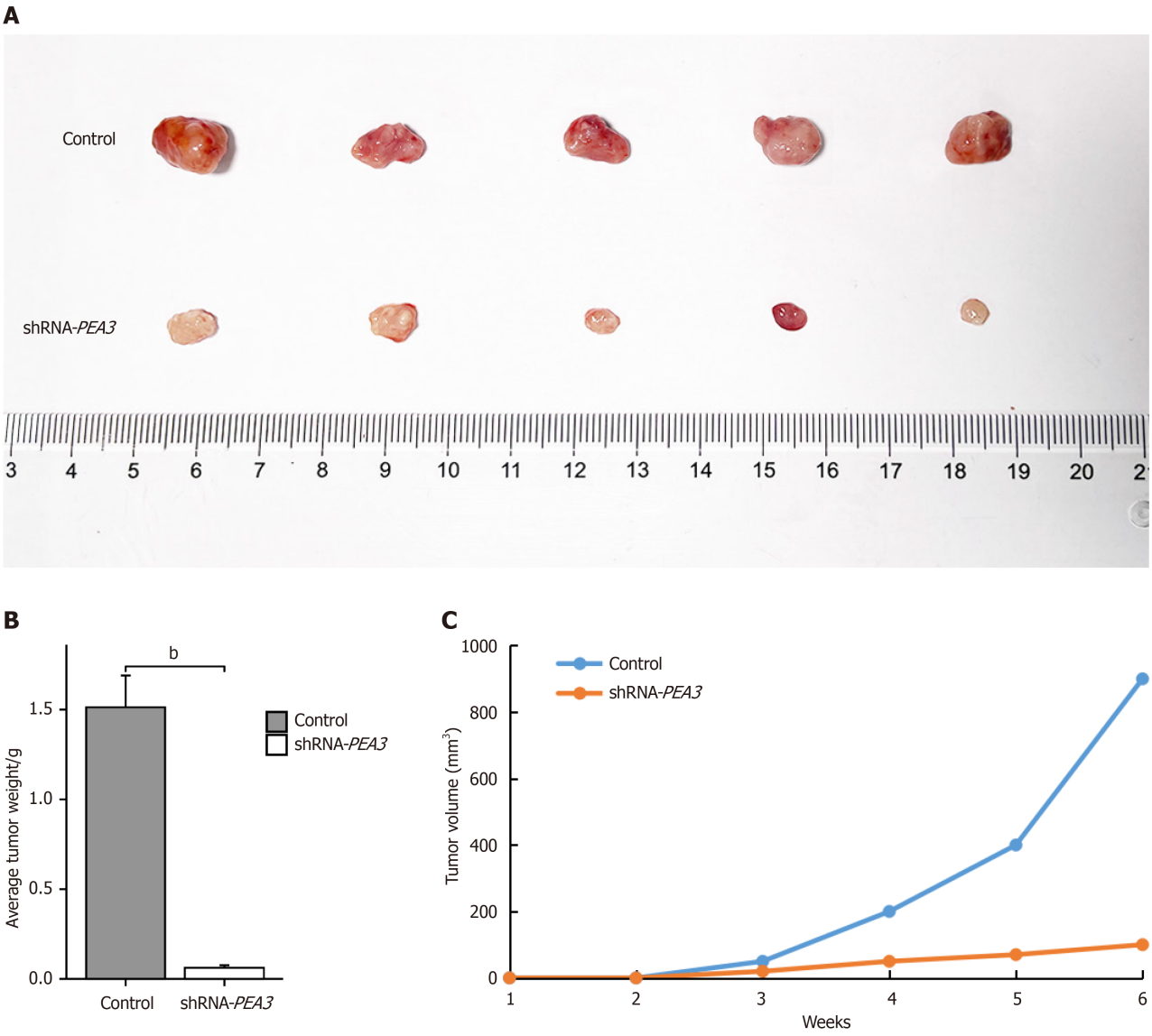

Figure 9 Inhibition of cholangiocarcinoma cell function after transfection with shRNA-*PEA3 in vivo*. A: Comparison of subcutaneous tumor weight; B: Statistics of subcutaneous tumor weight; C: The growth rate of subcutaneous tumor volume. $^bP < 0.01$.

CONCLUSION

In summary, our results revealed that *ETV1*, *ETV4*, and *ETV5* expression levels were significantly increased in CCA, and predictive analysis revealed that high *ETV4* expression levels were particularly related to shorter OS in CCA patients. These results suggest that *ETV4* expression levels can be prognostic biomarkers for CCA.

FOOTNOTES

Author contributions: Wang L analyzed the data and did all the experiments, writing-original drafted the manuscript; Zhang Z designed the project, methodology and funding acquisition; Zhang Z and Ma HZ designed the study, supervised the data collection together; All the authors revised and corrected the manuscript.

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