This study suggested that PES1 was upregulated in liver cancer and correlated with poor survival. We found that PES1 was highly expressed in liver cancer tissues, served as a diagnostic marker, and correlated with poor overall survival (OS) and relapse-free survival (RFS) in patients. In vitro studies indicated that PES1 promoted tumor cell proliferation (P=0.0034), migration (P=0.0026), and invasion (P=0.0008), and this tumorigenic role was confirmed in animal models. GSEA further illuminated molecular mechanisms that PES1 participated in.

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Conclusion: This study suggested that PES1 was upregulated in liver cancer and correlated with poor prognosis, by promoting tumor cell proliferation, migration, and invasion, and PES1 may be a novel diagnostic and prognostic bio-marker and a promising therapeutic target in liver cancer.

Keywords: liver cancer, PES1, diagnosis, tumorigenesis, prognosis

Introduction

Liver cancer has a high morbidity and mortality worldwide. Globally, it ranked sixth of all tumors for incidence and third for deaths in 2013, while in developing countries it ranked fifth for incidence and second for death.1 Despite rapid developments in medical technology, it remains an incurable disease that places a huge burden on patients and society.2 The poor prognosis of liver cancer can be attributed to the fact that most diagnoses currently take place in the late stage of disease progression.3 Therefore, the identification of a specific biomarker for early-stage diagnosis and prognosis has great clinical importance.
DNA replication and posttranscriptional modifications are necessary processes in tumor initiation and progression. Pescadillo (PES1) is a nuclear protein of 588 amino acids that mediates the protein transport process. It plays important roles in embryonic development, ribosome synthesis, DNA replication, and cell cycle progression. Recently, abnormal PES1 upregulation has been identified in some cancers, including neuroblastoma, colon cancer, gastric cancer, and breast cancer. The upregulation of PES1 in hepatocellular carcinoma (HCC) was reported by Fan et al in 2018 and Wang et al in 2019; however, the expression of PES1 in other liver cancer types, in distinct subgroups, and diagnostic values has not been revealed. Furthermore, as a tumorigenesis-related molecule, the role of PES1 in cell migration and invasion remain to be elucidated.

In this study, we examined the expression of PES1 in liver cancer and analyzed its relationship with patient prognosis. We also verified the tumorigenic function of PES1 in liver cancer in vitro and in vivo. Our results suggest that PES1 could be a novel diagnostic and prognostic biomarker in patients with liver cancer.

Materials And Methods
Bioinformatic Analysis

RNA sequencing and clinical data for liver cancer patients were downloaded from The Cancer Genome Atlas (TCGA) database using the RTCGAToolbox package in R (version 3.5.1). Gene Set Enrichment Analysis (GSEA) was performed using the GSEA 3.0 software and the enrichment score (ES) was calculated for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways from the Molecular Signature Database.

Cell Lines And Culture

Hepa 1–6 cells were purchased from the American Type Culture Collection (USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Solarbio). Cells were incubated in a humidified incubator with 5% CO₂.

Cell Transfection

The PES1 sequence was amplified and inserted into pCMV vector (Beyotime). The transfection of PES1 over-expression or control plasmid was performed using Lipofectamine 3000 (Invitrogen) following the manufacturer’s instructions. After 48 hrs, cells were collected and used for experiments.

RNA Isolation And PCR

RNA was extracted using Trizol Reagent (Invitrogen) from the transfected cells, according to the manufacturer’s instructions. cDNA was synthesized from 1000 ng of RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa). PCR was carried out according to the following procedures: 95°C 3 mins, followed by 35 cycles of 95°C 30 s, 58°C 30 s, and 72°C 2 mins. The primers used were listed as follows: PES1 forward primer: 5′- ATGGAGGTCTGGAGAAG-3′; PES1 reverse primer: TCACACGGCCTTGTCCCTTT.

Proliferation Assay

Hepa 1–6 cells were seeded in 96-well plates at a concentration of 1×104 cells per well and transfected with PES1 or EGFP plasmid. Forty-eight hrs after transfection, MTT (Sigma) was added to the cells and the absorption at 570 nm was measured 2 hrs later.

Migration Assay

Hepa 1–6 cells were grown in 6-well plates until confluent and transfected with PES1 or EGFP plasmid. Forty-eight hrs after transfection, cells were scratched with a p200 tip. Photographs were taken immediately and 24 hrs after scratching. The size of the scratches was measured by ImageJ software (NIH).

Invasion Assay

Transwell chambers (8 mm pore size) were coated with Matrigel (Corning) according to the manufacturer’s instructions. PES1- or EGFP-transfected cells were plated in the upper chambers in serum-free medium, while medium containing 10% FBS was used to fill the lower chambers. After 24 hrs incubation, cells were fixed with 4% paraformaldehyde and stained with Giemsa stain (Solarbio). The number of invading cells was counted in five random fields (×100 magnification) per insert.

In Vivo Experiments

The animal experiments were approved by the Institutional Animal Care and Use Committee of First Hospital of Jilin University and performed in accord with the institutional and NIH guidelines. 5×105 Hepa 1–6 liver cancer cells transfected with PES1 or EGFP control plasmid were injected subcutaneously into the flanks of C57BL/6 mice (8–12 weeks old, 18–22 g). Tumor volumes were...
measured every 2 days and calculated using the following formula: tumor volume = (length×width²)/2.

**Histology**
Animals were sacrificed and tumors were fixed in 4% paraformaldehyde (PFA) overnight and then embedded in paraffin. Sections at 4 μm were processed for Hematoxylin-Eosin (H&E) staining and images were photographed with a light microscope (Olympus).

**Statistical Analysis**
Differences between discrete variables were examined by box plots. The chi-squared test and Fisher’s exact tests measured the correlations between PES1 expression and liver cancer pathological features. Overall survival between different levels of PES1 expression was compared by Kaplan-Meier curves, with P-values calculated by the log rank test using the Survival package in R. Univariate Cox regression analysis was used to select potential variables associated with overall survival, while multivariate Cox analysis assessed the effects of PES1 expression on survival and other pathological features.

Data from in vitro and in vivo experiments were analyzed by the Student’s t-test (unpaired, two-tailed) using Graph Pad Prism Software. Results are shown as means ±SD. P<0.05 was considered statistically significant.

**Results**
**PES1 Is Upregulated In Liver Cancer**
Patient data were downloaded from the TCGA database. Of the 373 patients, 121 (32.44%) were female and 252 (67.56%) were male. A total of 244 (66.12%) patients...
were older than 55 years while 125 (33.88%) were younger. Detailed patient information is shown in Table 1.

The expression of PES1 was compared between patients with liver cancer and healthy controls (Figure 1A), and shown to be significantly elevated in liver cancer tissues (p=2.6e-07 by Wilcoxon test). The expression of PES1 in distinct stage of liver cancer (Figure 1B) was also analyzed, and it was found to be distinct (p=0.49 by Kruskal–Wallis test). PES1 expression was also found to be significantly increased as histological grade progressing (p=0.0072 by Kruskal–Wallis test) (Figure 1C). The deceased states of patients had higher PES1 expression level than living states (p=7e-04 by Wilcoxon test) (Figure 1D).

**Figure 1** Expression of PES1 mRNA in liver cancer based on analysis of TCGA data. (A) PES1 was significantly upregulated in liver cancer tissues when compared with normal liver tissues (p=2.6e-07 by Wilcoxon test). (B) The expression of PES1 in distinct stage of liver cancer (p=0.49 by Kruskal–Wallis test). (C) PES1 expression was increased as histologic grade progressing (p=0.0072 by Kruskal–Wallis test). (D) The deceased states of patients had higher PES1 expression level than living states (p=7e-04 by Wilcoxon test).

**PES1 Presents Modest Diagnostic Value In Liver Cancer**

Receiver operating characteristic (ROC) curve was generated to evaluate the diagnostic value of PES1. The area under the curve (AUC) was 0.724, which indicated moderate diagnostic value (Figure 2A). Subgroup analysis further demonstrated the diagnostic value of PES1 expression in distinct stage of liver cancer, with AUC of 0.718 for stage I (Figure 2B), 0.732 for stage II (Figure 2C),
Correlation Between Clinical Characteristics And PES1 Expression In Liver Cancer

The relationship between clinical features and expression of PES1 in patients with liver cancer is summarized in Table 2. Vital status (P=0.002067), sex (P=0.03887), and race (P=0.008083) were significantly correlated with PES1 expression.

Overexpression Of PES1 Is Associated With Poor Overall Survival (OS) In Patients With Liver Cancer

The role of PES1 expression in prognosis was analyzed by Kaplan–Meier analysis with the log rank test. Patients with higher PES1 expression had a significantly shorter survival time than those with lower expression (Figure 3A, P<0.0001). Subgroup analysis based on clinical stage found that PES1 upregulation significantly reduced OS in patients with both stage I/II (Figure 3B, P=0.007) and stage III/IV (Figure 3C, P=0.01). Univariate analysis revealed that poor OS was significantly associated with T classification (P=0.000), residual tumor (P=0.003), tumor stage (P=0.001), and PES1 expression (P=0.000). Multivariate tests using the Cox regression model revealed that high PES1 expression was an independent risk factor for patient prognosis (hazard ratio=2.09, P=0.000) (Table 3).

Overexpression Of PES1 Is Associated With Poor Relapse-Free Survival (RFS) In Patients With Liver Cancer

Kaplan-Meier analysis showed patients with higher PES1 expression presented shorter RFS than those with lower expression (Figure 4A, P=0.031). PES upregulation also impacted the RFS by subgroup analysis,
such as patients in stage I/II (Figure 4B, P=0.17) and stage III/IV (Figure 4C, P=0.095). Univariate analysis revealed poor RFS was associated with T classification (P=0.000), residual tumor (P=0.042), stage (P=0.000), and PES1 expression (P=0.031). Multivariate analysis further demonstrated high PES1 expression was an independent risk factor for patient prognosis (hazard ratio=1.44, P=0.032) (Table 4).

Table 2 Correlation Between The Clinical Variables And PES1 Expression In Patients

| Features          | Variables                  | N    | PES1 (%) | χ²   | P     |
|-------------------|----------------------------|------|----------|------|-------|
|                   |                            |      | High     | Low  |       |
| Vital status      | Survival                   | 243  | 107 (57.5)| 136 (72.7)| 9.489 | 0.002067 |
|                   | Death                      | 130  | 79 (42.5)| 51 (27.3)|       |        |
| T classification  | T1                         | 182  | 85 (45.7)| 97 (51.9)| 2.579 | 0.76461 |
|                   | T2                         | 95   | 50 (26.9)| 45 (24.1)|       |        |
|                   | T3                         | 80   | 43 (23.1)| 37 (19.8)|       |        |
|                   | T4                         | 13   | 7 (3.8) | 6 (3.2)  |       |        |
|                   | Tx                         | 1    | 0 (0.0) | 1 (0.5)  |       |        |
| N classification  | N0                         | 253  | 133 (71.5)| 120 (64.2)| 3.718 | 0.293629 |
|                   | N1                         | 4    | 1 (0.5) | 3 (1.6)  |       |        |
|                   | Nx                         | 187  | 52 (28.0)| 63 (33.7)|       |        |
| M classification  | M0                         | 267  | 135 (72.6)| 132 (70.6)| 0.188 | 0.910332 |
|                   | M1                         | 4    | 2 (1.1) | 2 (1.1)  |       |        |
|                   | Mx                         | 102  | 49 (26.3)| 53 (28.3)|       |        |
| Gender            | Female                     | 121  | 51 (27.4)| 70 (37.4)| 4.267 | 0.03887 |
|                   | Male                       | 252  | 135 (72.6)| 117 (62.6)|       |        |
| Radiation therapy | No                         | 341  | 166 (89.2)| 175 (93.6)| 2.322 | 0.3132 |
|                   | Yes                        | 9    | 6 (3.2) | 3 (1.6)  |       |        |
| Histological type | Fibrolamellar carcinoma    | 3    | 0 (0.0) | 3 (1.6)  | 3.143 | 0.207738 |
|                   | Hepatocellular carcinoma   | 363  | 182 (97.8)| 181 (96.8)|       |        |
|                   | Hepatocholangio-carcinoma (mixed) | 7  | 4 (2.2) | 3 (1.6)  |       |        |
| Residual tumor    | R0                         | 326  | 164 (88.2)| 162 (86.6)| 3.991 | 0.40729 |
|                   | R1                         | 17   | 8 (4.3) | 9 (4.8)  |       |        |
|                   | R2                         | 1    | 1 (0.5) | 0 (0.0)  | 14 (7.5)|       |
|                   | Rx                         | 22   | 8 (4.3) | 14 (7.5)|       |        |
| Race              | American Indian or Alaska native | 2  | 2 (1.1) | 0 (0.0)  | 13.765 | 0.008083 |
|                   | Asian                      | 159  | 95 (51.1)| 64 (34.2)|       |        |
|                   | Black or African American  | 17   | 7 (3.8) | 10 (5.3)|       |        |
|                   | White                      | 185  | 77 (41.4)| 108 (57.8)|       |        |
| Age               | ≤55                        | 125  | 64 (34.4)| 61 (32.6)| 4.332 | 0.114656 |
|                   | >55                        | 244  | 118 (63.4)| 126 (67.4)|       |        |
| Stage             | I                          | 172  | 83 (44.6)| 89 (47.6)| 2.016 | 0.732797 |
|                   | II                         | 87   | 48 (25.8)| 39 (20.9)|       |        |
|                   | III                        | 85   | 43 (23.1)| 42 (22.5)|       |        |
|                   | IV                         | 5    | 2 (1.1) | 3 (1.6)  |       |        |

Note: Bold values indicate P<0.05 and are statistically significant.

PES1 Promotes Liver Cancer Cell Proliferation, Migration, And Invasion In Vitro

Because of the association between PES1 upregulation and poor prognosis in liver cancer, we speculated that PES1 could promote tumor progression. We therefore overexpressed PES1 in liver cancer Hepa 1–6 cell lines and
investigated its effect on the cells. The MTT assay showed that PES1 upregulation significantly promoted Hepa 1–6 cell proliferation compared with control (P<0.01; Figure 5A). PES1 also increased the migratory distance of tumor cells in the scratch assay significantly (P<0.01; Figure 5B and C). Moreover, the invasion capacity was significantly increased in PES1-overexpressing cells (P<0.001; Figure 5D and E). Taken together, these results suggest that PES1 accelerated tumor progression by promoting tumor cell proliferation, migration, and invasion, and might be a therapeutic target for liver cancer.

PES1 Promotes Tumor Growth In Liver Cancer Models

As we found PES1 presented tumorigenic roles in vitro, we further investigated the effect of PES1 upregulation on tumor growth of Hepa 1–6 cells in C57BL/6 mice. Results showed tumors from PES1-overexpressing cells had a faster growth rate than tumors from control cells (P<0.05; Figure 6A). Meanwhile, the tumor weight from PES1-overexpressing cells was also higher than from control cells (P<0.01; Figure 6B). Histologic analysis further presented advanced degree of malignancy in PES1 overexpression tumors (Figure 6C).

Association Between KEGG Pathways And PES1 Expression

We explored and verified KEGG pathways which PES1 involved in the whole gene expression level using GSEA. As shown in Figure 7, five enriched KEGG pathways, including DNA replication, pyrimidine metabolism, ribosome, RNA polymerase, and spliceosome, were associated

Table 3 Association Between PES1 Expression And OS

| Characteristics         | Hazard Ratio | 95% CI       | P    | Hazard Ratio | 95% CI       | P    |
|-------------------------|--------------|--------------|------|--------------|--------------|------|
| PES1 expression         | 2.11         | 1.46–3.05    | 0.000| 2.09         | 1.44–3.02    | 0.000|
| T classification        | 1.66         | 1.39–1.99    | 0.000| 1.79         | 1.42–2.26    | 0.000|
| N classification        | 0.73         | 0.51–1.05    | 0.086|              |              |      |
| M classification        | 0.72         | 0.49–1.04    | 0.077|              |              |      |
| Gender                  | 0.80         | 0.56–1.14    | 0.220|              |              |      |
| Radiation therapy       | 0.51         | 0.26–1.03    | 0.060|              |              |      |
| Histological type       | 0.99         | 0.27–3.66    | 0.986|              |              |      |
| Residual tumor          | 1.42         | 1.13–1.80    | 0.003| 1.46         | 1.14–1.86    | 0.002|
| Histological grade      | 1.04         | 0.84–1.30    | 0.698|              |              |      |
| Age                     | 1.00         | 0.69–1.45    | 0.997|              |              |      |
| Stage                   | 1.38         | 1.15–1.66    | 0.001| 0.88         | 0.71–1.09    | 0.250|

Note: Bold values indicate P<0.05 and are statistically significant.
Abbreviation: CI, confidence interval.
with high PES1 expression, which further illuminated molecular mechanisms that PES1 participated in liver cancer occurrence and progression.

**Discussion**

In the present study, we determined the diagnostic and prognostic roles of PES1 expression in liver cancer patients and the functional consequences of PES1 in liver cancer cell line in vitro and in vivo. To our knowledge, no previous investigation has revealed the diagnostic value of the PES1 gene in human liver cancer, especially in subgroups, or the role of PES1 in migration and invasion of cells and RFS in patients. We concluded that PES1 was upregulated in liver cancer tissues, significantly associated with both OS and RFS. In vitro and in vivo experiments confirmed the tumorigenic effects of PES1, and GSEA also illustrated the molecular mechanisms that PES1 participated in.

A lack of diagnosis during early-stage disease contributes to the poor prognosis of liver cancer patients, so identifying a biomarker for early diagnosis would have notable benefits. DNA replication and posttranscriptional modifications play critical roles in tumorigenesis and tumor progression. As a nuclear protein, PES1 was first identified as essential for normal zebrafish embryonic development, and has been reported to play important roles in many other physiological processes including DNA replication, ribosome genesis, chromosomal stability, and cell proliferation. PES1 interacts with Bop1 and WDR12 to form the PeBoW-complex; this functions in the synthesis and maturation of ribosomes, which is a crucial process in cell proliferation. PES1 mutants or other disturbances to the complex can lead to p53 activation, which is regarded as the most common genetic alteration in human cancers, regulating numerous genes involved in the cell cycle, apoptosis, DNA repair, and metabolism.

**Table 4** Association Between PES1 Expression And RFS

| Characteristics | Hazard Ratio | 95% CI       | P     | Hazard Ratio | 95% CI       | P     |
|-----------------|--------------|--------------|-------|--------------|--------------|-------|
| PES1 expression | 1.44         | 1.03–2.01    | 0.031 | 1.44         | 1.03–2.02    | 0.032 |
| T classification| 1.78         | 1.49–2.12    | 0.000 | 1.60         | 1.22–2.09    | 0.001 |
| N classification| 0.97         | 0.67–1.40    | 0.874 | 0.97         | 0.66–1.43    | 0.432 |
| M classification| 1.17         | 0.79–1.74    | 0.432 | 1.17         | 0.79–1.74    | 0.432 |
| Gender          | 0.99         | 0.70–1.41    | 0.966 | 0.99         | 0.70–1.42    | 0.966 |
| Radiation therapy| 0.74        | 0.26–2.16    | 0.584 | 0.74         | 0.26–2.16    | 0.584 |
| Histological type| 2.02        | 0.66–6.24    | 0.220 | 2.02         | 0.66–6.24    | 0.220 |
| Residual tumor  | 1.28         | 1.01–1.61    | 0.042 | 1.36         | 1.07–1.71    | 0.011 |
| Histologic grade| 0.98        | 0.80–1.21    | 0.883 | 0.98         | 0.80–1.21    | 0.883 |
| Age             | 0.90         | 0.63–1.38    | 0.550 | 0.90         | 0.63–1.38    | 0.550 |
| Stage           | 1.66         | 1.38–1.99    | 0.000 | 1.16         | 0.89–1.50    | 0.273 |

**Note:** Bold values indicate P<0.05 and are statistically significant.

**Abbreviation:** CI, confidence interval.
Several studies have reported the upregulation of PES1 in tumor tissues. Xie et al reported significantly higher PES1 expression in colon cancer tissues compared with adjacent tissues, while it was also highly up-regulated in gastric cancers. Our study observed increased PES1 expression in liver cancer, which is in accordance with previous work. Additionally, we documented differences in PES1 expression in distinct clinical stages, histological grade, and vital statuses of patients, which further divided patients into subgroups. ROC analysis further provided the diagnostic value of PES1 expression in liver cancer.

PES1 overexpression is correlated with tumor initiation and proliferation. Short hairpin RNA-mediated PES1 ablation previously inhibited colon cancer cell proliferation, and Li et al reported that PES1 silencing inhibited gastric cancer growth and altered the expression of proliferation-related genes. Moreover, Fan et al found that PES1 overexpression promoted liver cancer cell proliferation while its knockdown inhibited their growth. Based on these studies, we propose that the correlation between PES1 and the vital status of patients reflects the role of PES1 in promoting tumor proliferation.

Several articles have reported the adverse effects of PES1 in cancer prognosis. In neuroblastoma, high PES1 expression was associated with aggregation and a worse outcome, while PES1 knockdown suppressed tumor growth and induce apoptosis. Additionally, PES1 was shown to regulate the balance between estrogen receptor (ER)α and ERβ to promote the progression of breast cancer and ovarian cancer. Similar to earlier findings, our study found that higher PES1 expression was associated with worse outcomes in patients with liver cancer; however, we also reported heterogeneity in the prognostic role of PES1 because of distinct clinical stages, which provide the possibility of precision therapy for patients with liver cancer. Moreover, we showed that PES1 was associated with poor OS in stage I/II,

![Figure 5](image_url) PES1 promoted liver cancer cell proliferation, migration and invasion. (A) MTT proliferation assay in PES1 or control plasmid transfected Hepa 1–6 cells. (B, C) Wound scratch assay showed PES1 overexpression promoted the migration of Hepa 1–6 cells. (D, E) Transwell invasion assay revealed PES1 overexpression cells had stronger invasion capacity than control. Error bars indicate the mean ± SD. **p < 0.01, ***p < 0.001.
suggesting that its prognostic evaluation role is effective even in the very early stages of the disease.

The MTT assay is one of the most common strategies to analyze cell proliferation. Our results showed that cells with upregulated PES1 exhibited a higher growth rate, suggesting that PES1 promotes tumor growth. The scratch assay and Transwell assay also revealed that PES1 upregulation increased cell migration and invasion, which further confirmed its strong correlation with tumor progression and metastasis. Finally, the tumorigenic roles of PES1 were verified in animal models, which made the theory more persuasive. Recently, two studies of the proliferative role of PES1 in hepatocellular carcinoma (HCC) obtained similar findings to our own research, which not only confirmed our results, but also suggested that PES1 was a research hotspot. Fan et al showed PES1

![Figure 6](image)

**Figure 6** PES1 promoted tumor growth in liver cancer models. Overexpression of PES1 increased tumor volume (A), tumor weight (B) and histological grade (C). Bar = 50 μm. Error bars indicate the mean ± SD. *p < 0.05, **p < 0.01.
was upregulated in HCC, promoted HCC proliferation and glycolysis. Wang et al revealed upregulated PES1 promoted HCC proliferation via PI3K/AKT pathway, and correlated worse OS. In the present study, we demonstrated PES1 was upregulated in all liver cancer patients, rather than HCC, and the expression was distinct in patients of subgroups. We presented PES1 could be a diagnostic marker in liver cancer, and this value was different in each stage. Besides OS, we also analyzed the RFS of patients and showed PES1 was an independent risk factor for prognosis evaluation in both OS and RFS. In addition to proliferation, cell migration and invasion were important factors for tumorigenesis, which was also assessed in our study. Moreover, GSEA provided the
pathways that PES1 participated in, which illuminated the molecular mechanisms of liver cancer occurrence and progression. However, all our data of patients were derived from the TCGA database; so, clinical samples are required to verify our findings in the future.

**Conclusion**

In conclusion, we found that PES1 was upregulated in liver cancer tissues and that its high expression was strongly correlated with clinical progression and poor survival in patients. In vitro experiments showed that PES1 promoted tumor growth, migration, and invasion, and animal models confirmed its tumorigenic roles. Our results suggest that PES1 could be a novel biomarker for the diagnosis and prognosis of patients with liver cancer and a promising target for tumor therapy.

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**Disclosure**

The authors report no conflicts of interest in this work.

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