Research Article

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The pseudogene PTTG3P promotes cell migration and invasion in esophageal squamous cell carcinoma

https://doi.org/10.1515/med-2019-0057
received March 12, 2019; accepted May 23, 2019

Abstract: Pseudogenes are pivotal funtional non-coding RNAs in tumorigenesis. Cumulative evidences have shown that pituitary tumor-transforming 3, pseudogene (PTTG3P), serves as an oncogene in multiple human cancers. However, its expression pattern, biological function, and potential targets in esophageal squamous cell carcinoma (ESCC) remain unknown. Here, by quantitative real-time polymerase chain reaction (qRT-PCR) in 50 cases of ESCC, we found that the expression of PTTG3P, PTTG1 and PTTG2 in esophageal squamous cancer tissues and cell lines were significantly higher than their normal counterparts ($P<0.01$). Spearman correlation analysis showed that the PTTG3P expression was positively correlated with the PTTG1 and PTTG2 expression in ESCC tissue samples ($P<0.05$). Additionally, the high expression of PTTG3P in ESCC was significantly correlated with tumor depth, lymph node invasion and TNM stage ($P<0.05$). We also assessed the function of PTTG3P in vitro by gain-of-function studies. Results showed that enhanced expression of PTTG3P stimulated the migration and invasion of ESCC cells, and promoted the expression level of PTTG1 and PTTG2 in vitro. Furthermore, PTTG3P fulfilled its oncogenic functions by positively regulating its parent gene PTTG1 and PTTG2. Overall, our study indicated that PTTG3P is distinctly overexpressed and exhibited oncogenic role in a PTTG1 and PTTG2 mediated manner in ESCC.

Keywords: Pseudogene; PTTG3P; Esophageal squamous cell carcinoma; Regulation; Cell metastasis

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1 Introduction

In recent years, it has been found that many genes can be transcribed into non-coding regulatory RNAs (ncRNA). Although ncRNAs can't function biologically by encoding proteins, they have been shown to play important roles in a diverse range of cellular functions, such as cell proliferation, differentiation and development [1]. Pseudogenes are a type of non-coding RNA associated with the true (parent) gene (functional gene) that is widely present in human entities [2]. Pseudogene typically exhibit a high similarity to its known true gene while loss of certain function. That is to say, although each pseudogene has a DNA sequence similar to its parent gene, the pseudogene has lost some functions related to the complete gene in terms of cellular gene expression or protein coding. However, in recent years, a great deal of literature has shown that "pseudo" in pseudogenes only indicates changes in the sequence relative to the true coding gene, but doesn't mean "pseudo" in function [2, 3]. Many pseudogenes have been emerged as essential regulators in various of physiological processes and even in tumors [4-7].

The pseudogene PTTG3P (pituitary tumor-transforming 3, pseudogene) is an intronless gene highly homologous to PTTG1 (pituitary tumor-transforming 1) and PTTG2 (pituitary tumor-transforming 2). It can be expressed in a variety of normal adult tissues [8]. In human cancer entities, PTTG3P is specifically expressed in gastric cancer and hepatocellular carcinoma relative to normal human stomach and liver tissues [9, 10]. Moreover, the high expression of PTTG3P mRNA promoting cancer development by regulating the expression of its true gene PTTG1 mRNA in hepatocellular carcinoma [10]. However, there is no report on the expression of PTTG3P and its effects on PTTG1 and PTTG2 in esophageal squamous cell carcinoma (ESCC). In the present study, we intend to observe the differential expression and correlation of PTTG3P, PTTG1 and PTTG2 in ESCC, and further analyze the effect of PTTG3P on the expression of PTTG1 and PTTG2 in esophageal

cancer cells, as well as the potential role of PTTG3P on ESCC metastasis.

2 Materials and methods

2.1 Patient samples

A total of 50 ESCC patients were enrolled in this study. Fresh ESCC samples and adjacent non-tumor tissues were obtained from patients who had undergone routine surgery from 2016 to 2018 at Danyang People's hospital. Tissues were frozen and stored in liquid nitrogen until further use. Medical records of all patients provided information of age, gender, and following parameters: tumor size, differentiation, infiltration depth, lymph node metastasis, distant metastasis and TNM stage. Written informed consent was obtained from all participants, and the study was approved by the Ethics Committee of Danyang People's Hospital.

2.2 Cell lines and culture conditions

The immortalized human esophageal epithelial cell line (HEEC) and the malignant esophageal carcinoma cell lines (KYSE150, KYSE180, KYSE450, and KYSE140) were gifts from Professor Xiaoshan Feng (The First Affiliated Hospital of Henan University of Science and Technology). All cells were grown and maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA), maintain at 37°C in a humidified atmosphere with 5% CO_2.

2.3 Cell transfection

For reagents used: Namipo™ transfection reagent was purchased from Shanghai Quanyang Biotechnology Co., Ltd. The pcDNA3.1-PTTG3P and empty vector (used as a negative control), the siRNA of PTTG3P, PTTG2, PTTG1 and scramble siRNA, were all purchased from GeneChem, Shanghai, China. Cells were seeded in 24- or 6-well plates 24h before the experiment. KYSE140 cells were transfected with pcDNA3.1-PTTG3P or empty vector. KYSE180 cells were transfected with siRNA or scramble siRNA.

2.4 Cell migration and invasion detection

The transwell assay was used to assess cell migration and invasion with the transwell system of Corning co. Ltd., USA. Inserts containing 8-μm pore filters were uncoated for the migration assays or coated with Matrigel (Becton-Dickinson Labware, Bedford, MA) for the invasion assays. A total of 1.5×10^5 cells transfected with pcDNA3.1-PTTG3P or pcDNA3.1 in 100 μl of serum-free medium were added to each well. After 24 h of incubation at 37 °C, the cells that had migrated through the filters were fixed with methanol, stained with Giemsa and counted from 10 random fields under a microscope at 400× magnification. Each experiment was performed in triplicate.

2.5 Total RNA isolation and RT-qPCR

Total RNA was isolated from tissues and cells by Trizol (Invitrogen, Life technology, USA). The PCR reaction conditions were described as followed: initially denatured at 94° for 3min; denatured at 94° for 30s, annealed at 55° for 30s, extended at 72° for 30s, this procedure was repeated 30 cycles; and finally extended at 72° for 10min. GAPDH was used as an endogenous control to normalize the data. Primer sequences used in this study were the following: 5'- AAACGAAGAACCAGGCATCCTT -3' (forward) and 5'-GGGAGCATCGAATGTTTGCC-3' (reverse) for PTTG3P, 5'-TGACTGTTCCGCTGTTTAGC-3' (forward) and 5'-TAAGGCTTTGATTGAAGGTCCAG-3' (reverse) for PTTG1, 5'-ATTGGAGAACCAGGCACC-3' (forward) and 5'-CGTCGTGTTAAAACTTGAGATA-3' (reverse) for PTTG2, 5'-CATGGCCTTCCGTGTTCCTA-3' (forward) and 5'-TGTCATCATACTTGGCAGGTTT-3' (reverse) for GAPDH.

2.6 Western blot analysis

Western blotting was performed using a SDS-PAGE Electrophoresis System with antibodies specific for PTTG1(1:100, Proteintech. USA), PTTG2 (1:1000, Cell Signaling Technology) and β-actin (1:5000, Proteintech. USA). After washed with phosphate-buffered saline (PBS; Solarbio®, Beijing, China) three times, the secondary antibodies IgG H&L (Abcam, Cambridge, MA, USA) and IgG H&L (Abcam, Cambridge, MA, USA). Subsequently, the membranes were washed three times with phosphate-buffered saline (PBS; Solarbio®, Beijing, China), containing 0.1% Tween 20 (Sigma-Aldrich, Shanghai, China). The results were visualized using the X-ray films after adding 200 μl chemiluminescent system (Thermo Fisher Scientific, Inc.,

Waltham, MA, USA). β-actin functioned as the reference protein.

2.7 Statistical analysis

SPSS 18.0 statistical software (IBM Corporation, Armonk, NY, USA) was used to analyze the data. The data were presented as the mean ± deviation. Student's t-test was used to determine differences between two groups, while one-way analysis of variation (*ANOVA*) followed by Kruskal–Wallis analysis was applied to verify differences among groups. Spearman chi-square tests used were used for correlation analysis between PTTG3P levels and clinical features. Spearman test were used for correlation analysis of gene expression. A P value <0.05 was considered to be statistically significant.

3 Results

3.1 The expression of PTTG3P is up-regulated in ESCC and its level is positively correlated with PTTG1 and PTTG2 expression

As shown in Figure 1, the expression of PTTG3P mRNA in ESCC tissues was significantly higher than that in adjacent tissues (p = 0.005); moreover, the relative expression of PTTG1 mRNA in ESCC tissues was significantly higher than that in adjacent tissues (p < 0.001), and the relative expression of PTTG2 mRNA in ESCC was also significantly higher than para-cancerous tissues (p < 0.001). Spearman test was used to analyze the relationship between PTTG3P, PTTG1 and PTTG2 gene expression in ESCC tissue samples. The results showed that PTTG3P gene expression was significantly positively correlated with PTTG1 (r = 0.391, P = 0.005) and PTTG2 (r = 0.516, P < 0.001; Figure 2).

The level of PTTG3P in four ESCC cell lines (KYSE150, KYSE180, KYSE450, and KYSE140) and human esophageal epithelial cell line HEEC was also detected by qRT-PCR, which showed that PTTG3P expression was significantly elevated in the ESCC cell lines (Figure 3).

3.2 High PTTG3P expression is positively associated with high tumor burden in patients with ESCC

When the PTTG3P expression divided into high expression group and low expression group by the median value (1.272), the expression of PTTG3P in esophageal cancer was significantly associated with infiltration depth ($\chi 2$ = 0.205, P = 0.017), lymph node metastasis ($\chi 2$ = 0.147, P = 0.025) and tumor TNM staging ($\chi 2$ = 0.399, P = 0.039). However, the expression of PTTG3P was not associated with age, gender, degree of differentiation and tumor size (P > 0.05).

3.3 PTTG3P-overexpressing promotes ESCC cell migration and invasion in *vitro*

Given that the expression of PTTG3P in esophageal cancer was significantly associated with infiltration depth and lymph node metastasis, to determine whether PTTG3P promotes ESCC cell invasion and migration, we performed transwell chamber assays after determined the effert of PTTG3P pcDNA3.1 plasmid and siRNA (Figure 4A). Transwell chamber assays showed that overexpression of PTTG3P resulted in significantly increased migratory potential in KYSE140 cells compared with control cells, while knockdown of PTTG3P resulted in significantly

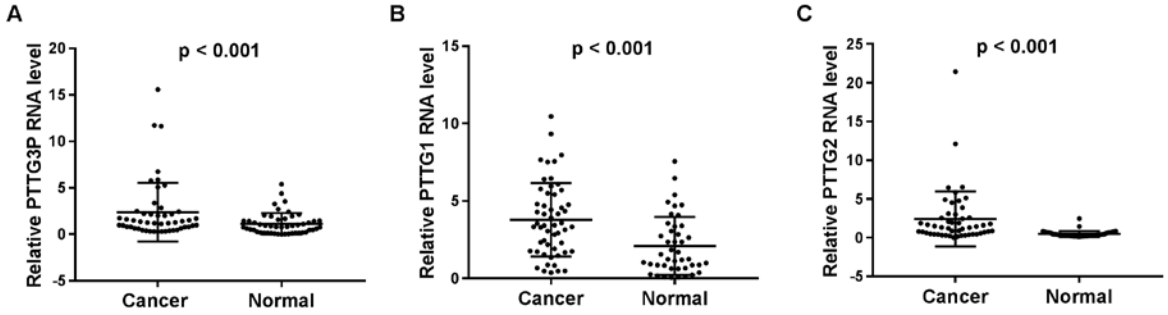

Figure 1: PTTG3P, PTTG1 and PTTG2 expression in esophageal cancer and adjacent normal tissues

A-C. Expression of PTTG3P, PTTG1 and PTTG2 mRNA in esophageal cancer tissues and adjacent normal tissues were detected by qRT-PCR. GAPDH was used as an internal control. Data are shown as the mean ± SD of three replicates.

Table 1: Relationship between PTTG3P expression and clinicopathological features of ESCC

Baseline variables	Number of patients	PTTG3P expression level		P value
		Low (n=25) No.	High (n=25) No.	
Age	50	62.31±19.36	59.85±21.39	0.163
Gender				0.634
Man	35	14	21	
Female	15	6	9	
Differentiation				0.128
High	18	6	12	
Medium-low	32	15	17	
Tumor size				0.610
≤4.0 cm	21	15	6	
>4.0 cm	29	16	13	
Tumor depth				0.017*
T1	19	12	7	
T2-3	31	15	16	
Lymph node metastasis				0.025*
Absent	29	15	14	
Present	21	9	12	
TNM stage				0.039*
I+II	23	7	16	
III	27	15	12	

* P < 0.05

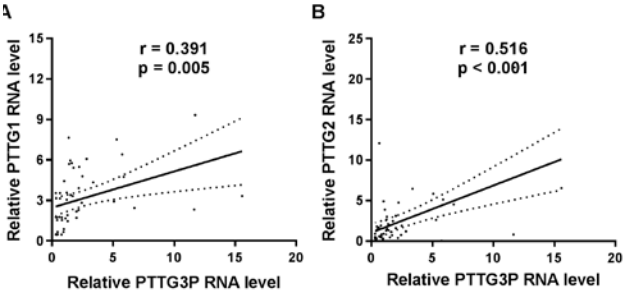

Figure 2: PTTG3P expression is positive correlated with PTTG1 and PTTG2 expression.

A-B. Correlations between PTTG3P mRNA expression and PTTG1 or PTTG2 mRNA expression were evaluated by Spearman correlation test.

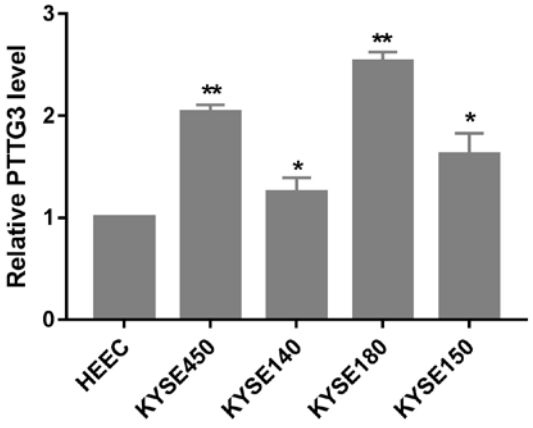

Figure 3: Baseline PTTG3P expression in ESCC cell lines and normal esophageal cell.

PTTG3P mRNA expression was quantitated in 4 ESCC cell lines and the HEEC cell line using qRT–PCR. GAPDH was used as an internal control. Data are shown as the mean ± SD of three replicates. *P<0.05; ** P<0.01.

decreased migratory potential in KYSE180 cells compared with control cellss; For invasion assays, the cells were detached and subsequently added onto Matrigel-coated Transwell chambers. Quantification of cells which had invaded through Matrigel, showed that overexpression of

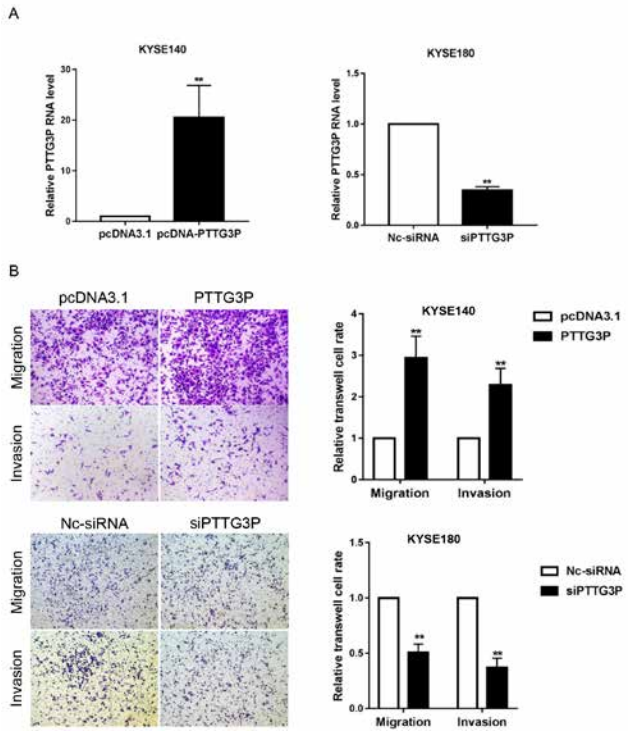

Figure 4: PTTG3P promoted cell invasion and migration in ESCC.

A. mRNA expression levels of PTTG3P in PTTG3P-overexpressing KYSE140 cells and PTTG3P-knockdown KYSE180 cells were evaluated by qRT–PCR, GAPDH was used as an internal control; B. Representative images of Transwell migration and invasion assays for KYSE140 cells (Scale bars = 50μm); and quantification (the ratio of cell number in the experimental group and the control group) of Transwell migration and invasion assays for KYSE140 cells. Data are shown as the mean ± SD of three replicates. ** P<0.01.

PTTG3P increased the number of invaded cells in KYSE140 cells, while knockdown of PTTG3P resulted in significantly decreased invasiveness of KYSE180 cells compared with control cells (Figure 4B). Thus, these data suggest that lncRNA PTTG3P induces ESCC cell migration and invasion in *vitro*.

3.4 PTTG3P facilitates tumor migration and invasion in by mediated PTTG1 and PTTG2

Finally, we conducted in *vitro* experiments to investigate whether PTTG3P functioned by mediating PTTG1 and PTTG2 in ESCC after determined the knockdown effert of PTTG1 and PTTG2 siRNA in KYSE180 cells (Figure 6A). Cell Transwell assays showed that PTTG1 and PTTG2 knockdown partially attenuated the effects of PTTG3P-overexpressing on ESCC cell migration and invasioncompared with that of the controls (Figure 6B). Our results indicated

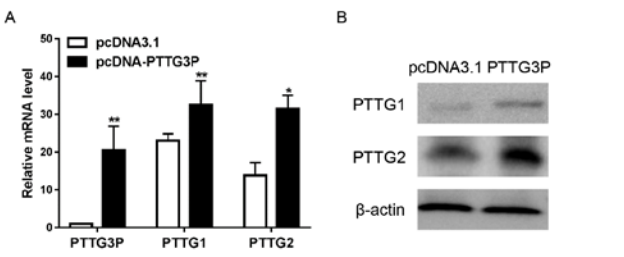

Figure 5: Effect of overexpression of PTTG3P on PTTG1 and PTTG2 in KYSE140 cells

A. mRNA expression levels of PTTG1 and PTTG2 in PTTG3P-overexpressing cells were evaluated by qRT–PCR, GAPDH was used as an internal control; B. Protein expression levels of PTTG1 and PTTG2 in PTTG3P-overexpressing cells were evaluated by Western blotting, β-actin was used as an internal control; Data are shown as the mean ± SD of three replicates; *P<0.05; ** P<0.01.

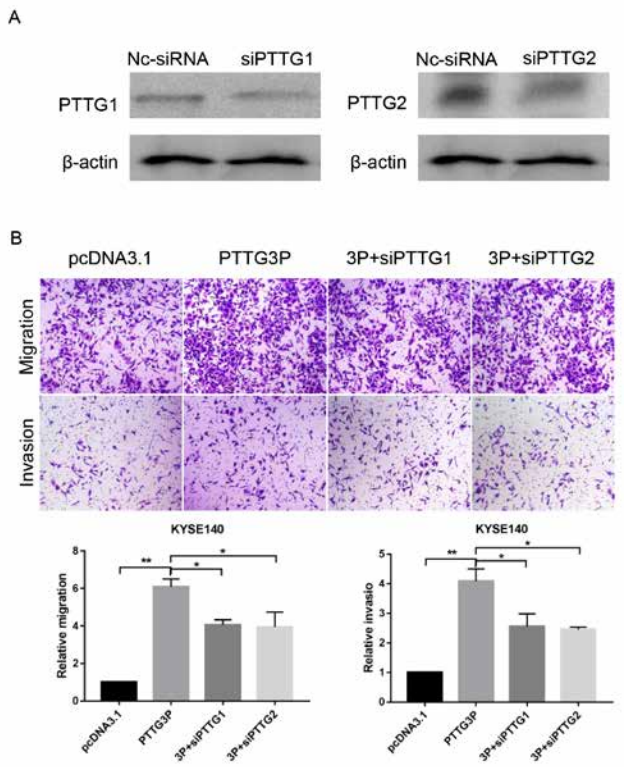

Figure 6: Effect of knockdown of PTTG1 and PTTG2 in PTTG3P-overexpressing KYSE140 cells

A. Protein expression levels of PTTG1 and PTTG2 in siRNA transfected KYSE140 cells were evaluated by Western blotting, β-actin was used as an internal control; B. Representative images (upper) and the number of migratory cells (down) per high-power field showed that knockdown of PTTG1 and PTTG2 partially attenuated the enhanced migratory and invasive ability of KYSE140 cells promoted by PTTG3P upregulation. Data are shown as the mean ± SD of three replicates; *P<0.05; ** P<0.01.

that PTTG3P promotes ESCC cell migration and invasion by mediating PTTG1 and PTTG2 .

4 Discussion

In the past ten years, pseudogenes have gained more and more attentions because of their important roles in human malignancies [11]. A pseudogene database, named dreamBase, has been developed to comprehensive display the expression level, DNA methylation modification, RNA regulation and RNA binding proteins of pseudogenes in human tumor entities [12]. At present, the function and mechanism of some pseudogenes in esophageal cancer have been clarified. For example, the expression of PTEN pseudogene PTENP1 in esophageal cancer cells is significantly lower than that in normal esophageal epithelial cells, and its differential expression is significantly correlated with tumor histological grade, TNM stage and prognosis; moreover, by complementary binding miR-17-5p, PTENP1 mediated the SOCS6/p-STAT3/HIF-1α signaling pathway and inhibited the proliferation of esophageal cancer cells [13]. The 3'UTR region of the pseudogene PHBP1 can acted as a ceRNA and upregulated its true gene PHB, thus promoted the proliferation and metastasis of esophageal cancer cells[14]. Obviously, pseudogenes play important roles in the development of esophageal cancer and have great research prospects.

In 2000, Kakar et al. [8] found a pseudogene PTTG3P that is highly similar to the PTTG1 and PTTG2 genes, but lacks introns in the gene sequence. Subsequently, PTTG3P was found dysregulated in gastric cancer [9] and hepatocellular carcinoma [10]. By detecting 50 cases of ESCC tissues and adjacent normal esophageal tissues in this present study, we reported for the first time that PTTG3P expression was significantly higher in ESCC tissues and cell lines than that in their normal counterparts. These results suggest that PTTG3P can also be expressed in esophageal malignancies.

Lots of pseudogene RNA actually has a "true" function in life: some pseudogenes can be used as antisense RNA to bind to their true gene, or processed into endo-siRNAs to inhibit true gene expression; some pseudogene RNA can acted as ceRNA, binding with miRNA and modulating true genes; besides, some pseudogene RNA regulate RNA expression or protein translation through interact with RNA binding proteins or translational machinery [15]. Previous studies have confirmed that PTTG3P is a biologically functional gene: Weng et al [9] reported that PTTG3P can promote tumor cell proliferation and metastasis in gastric cancer, whereas it has no effect on the expression of its true gene PTTG1 and PTTG2. However, Huang et al [10] found that PTTG3P can promote tumor malignant phenotype by up-regulating PTTG1 expression and mediating PI3K/AKT signaling pathway in hepatocellular carcinoma. Our study found that, similarly with PTTG3P, the expression of PTTG1 and PTTG2 in esophageal cancer tissues were also significantly higher than that in normal esophageal tissues. Also, the expression of PTTG3P was positively correlated with that of PTTG1 and PTTG2 in ESCC tissues, suggesting PTTG3P may also regulate the transcription of PTTG1 and PTTG2 genes in ESCC. We further confirmed that PTTG1 and PTTG2 expression levels in PTTG3P-overexpressing KYSE140 cells were 1.41 and 2.28 times higher than those of control cells, respectively. Our results suggest that exogenous overexpression PTTG3P may promote the transcription of its parental genes PTTG1 and PTTG2, and further in *vitro* experiments validated this hypothesis. However, the significance of this regulatory mechanism for the development of esophageal cancer deserve further study.

In summary, the current study suggests that PTTG3P exhibits an oncogenic role in ESCC. PTTG3P promotes cell migration and invasion by positively regulates its parent genes PTTG1 and PTTG2. Our results provide new insights into the molecular details of pseudogene in ESCC.

Conflict of interest statement: The authors declare that they have no conflicts of interest.

References

[1] Mattick J.S. The genetic signatures of noncoding RNAs. PLoS Genet. 2009, 5(4):e1000459. DOI: 10.1371/journal.pgen.1000459

[2] Poliseno L., Salmena L., Zhang J., Carver B., Haveman W.J. and Pandolfi P.P. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature. 2010, 465(7301):1033-1038. DOI: 10.1038/nature09144

[3] Chan W.L., Yuo C.Y., Yang W.K., Hung S.Y., Chang Y.S., Chiu C.C., et al. Transcribed pseudogene psiPPM1K generates endogenous siRNA to suppress oncogenic cell growth in hepatocellular carcinoma. Nucleic Acids Res. 2013, 41(6):3734-3747. DOI: 10.1093/nar/gkt047

[4] Hayashi H., Arao T., Togashi Y., Kato H., Fujita Y., De Velasco M.A., et al. The OCT4 pseudogene POU5F1B is amplified and promotes an aggressive phenotype in gastric cancer. Oncogene. 2015, 34(2):199-208. DOI: 10.1038/onc.2013.547

[5] Karreth F.A., Reschke M., Ruocco A., Ng C., Chapuy B., Leopold V., et al. The BRAF pseudogene functions as a competitive endogenous RNA and induces lymphoma in vivo. Cell. 2015, 161(2):319-332. DOI: 10.1016/j.cell.2015.02.043

[6] Mattoo A.R., Zhang J., Espinoza L.A. and Jessup J.M. Inhibition of NANOG/NANOGP8 downregulates MCL-1 in colorectal cancer cells and enhances the therapeutic efficacy of BH3 mimetics. Clin Cancer Res. 2014, 20(21):5446-5455. DOI: 10.1158/1078-0432.CCR-14-1134

[7] Puget N., Gad S., Perrin-Vidoz L., Sinilnikova O.M., Stoppa-Lyonnet D., Lenoir G.M., et al. Distinct BRCA1 rearrangements involving the BRCA1 pseudogene suggest the existence of a recombination hot spot. Am J Hum Genet. 2002, 70(4):858-865. DOI: 10.1086/339434

[8] Chen L., Puri R., Lefkowitz E.J. and Kakar S.S. Identification of the human pituitary tumor transforming gene (hPTTG) family: molecular structure, expression, and chromosomal localization. Gene. 2000, 248(1-2):41-50.

[9] Weng W., Ni S., Wang Y., Xu M., Zhang Q., Yang Y., et al. PTTG3P promotes gastric tumour cell proliferation and invasion and is an indicator of poor prognosis. J Cell Mol Med. 2017, 21(12):3360-3371. DOI: 10.1111/jcmm.13239

[10] Huang J.L., Cao S.W., Ou Q.S., Yang B., Zheng S.H., Tang J., et al. The long non-coding RNA PTTG3P promotes cell growth and metastasis via up-regulating PTTG1 and activating PI3K/AKT signaling in hepatocellular carcinoma. Mol Cancer. 2018, 17(1):93. DOI: 10.1186/s12943-018-0841-x

[11] Hu X., Yang L. and Mo Y.Y. Role of Pseudogenes in Tumorigenesis. Cancers (Basel). 2018, 10(8). DOI: 10.3390/cancers10080256

[12] Zheng L.L., Zhou K.R., Liu S., Zhang D.Y., Wang Z.L., Chen Z.R., et al. dreamBase: DNA modification, RNA regulation and protein binding of expressed pseudogenes in human health and disease. Nucleic Acids Res. 2018, 46(D1):D85-d91. DOI: 10.1093/nar/gkx972

[13] Gong T., Zheng S., Huang S., Fu S., Zhang X., Pan S., et al. PTENP1 inhibits the growth of esophageal squamous cell carcinoma by regulating SOCS6 expression and correlates with disease prognosis. Mol Carcinog. 2017, 56(12):2610-2619. DOI: 10.1002/mc.22705

[14] Zheng L., Li X., Gu Y., Lv X. and Xi T. The 3'UTR of the pseudogene CYP4Z2P promotes tumor angiogenesis in breast cancer by acting as a ceRNA for CYP4Z1. Breast Cancer Res Treat. 2015, 150(1):105-118. DOI: 10.1007/s10549-015-3298-2

[15] Xu Y., Yu X., Wei C., Nie F., Huang M. and Sun M. Over-expression of oncigenic pesudogene DUXAP10 promotes cell proliferation and invasion by regulating LATS1 and beta-catenin in gastric cancer. J Exp Clin Cancer Res. 2018, 37(1):13. DOI: 10.1186/s13046-018-0684-8