

## Original Article

# A pan-cancer analysis of pituitary tumor-transforming 3, pseudogene

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**Abstract:** Background: Although evidence regarding pituitary tumor-transforming 3, pseudogene (PTTG3P) involvement in human cancers has been acquired via human and animal model-based molecular studies, there is a lack of pan-cancer analysis of this gene in human tumors. Methods: Tumor-causing effects of PTTG3P in 24 human tumors were explored using The Cancer Genome Atlas (TCGA) datasets from different bioinformatics databases and applying in silico tools such as The University of Alabama at Birmingham CANcer (UALCAN), Human Protein Atlas (HPA), Kaplan Meier (KM) plotter, cBioPortal, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), Cytoscape, Database for Annotation, Visualization, and Integrated Discovery (DAVID), Tumor IMMune Estimation Resource (TIMER), and Comparative Toxicogenomics Database (CTD). Then, via in vitro experiments, including RNA sequencing (RNA-seq) and targeted bisulfite sequencing (bisulfite-seq), expression and promoter methylation levels of PTTG3P were verified in cell lines. Results: The PTTG3P expression was overexpressed across 23 malignancies and its overexpression was further found significantly effecting the overall survival (OS) durations of the esophageal carcinoma (ESCA) and head and neck cancer (HNSC) patients. This important information helps us to understand that PTTG3P plays a significant role in the development and progression of ESCA and HNSC. As for PTTG3P functional mechanisms, this gene along with its other binding partners was significantly concentrated in "Oocyte meiosis", "Cell cycle", "Ubiquitin mediated proteolysis", and "Progesterone-mediated oocyte maturation". Moreover, ESCA and HNSC tissues having the higher expression of PTTG3P were found to have lower promoter methylation levels of PTTG3P and higher CD8+ T immune cells level. Additionally, PTTG3P expression-regulatory drugs were also explored in the current manuscript for designing appropriate treatment strategies for ESCA and HNSC with respect to PTTG3P expression. Conclusion: Our pan-cancer based findings provided a comprehensive account of the oncogenic role and utilization of PTTG3P as a novel molecular biomarker of ESCA and HNSC.

**Keywords:** Pan-cancer, PTTG3P, cancer, biomarker

### Introduction

Cancer is a killer malignancy and so far its accurate detection is still a challenging task [1-3]. Moreover, the occurrence frequency of this disease is reported to be higher than its cure rate worldwide [4, 5]. Recent studies have

highlighted that cancer occurrence is a complex process and is directly related to the expression variations in several onco and proto-oncogenes that ultimately result in the alteration of important signaling pathways [6, 7]. Despite significant improvements in diagnosis, predicting prognosis, and treatment methods, the progno-

sis of cancer patients is still dismal [8-10]. Therefore, the discovery of more effective biomarkers may help to detect cancer more accurately and improve the prognosis. As the tumor genesis process and the development of cancer are mechanistically very complex, numerous genes could play key roles in developing cancer, thus, it is very important to analyze any cancer-associated significant genes' via pan-cancer analysis and assess the correlations between gene expression and clinical prognosis [11, 12].

Pseudogenes include non-coding RNAs and DNA segments related to functional genes [13]. Based on formation mechanisms, pseudogenes have been divided into 3 subtypes: unitary pseudogenes, processed pseudogenes, and duplicated pseudogenes [13, 14]. Pseudogene dysregulation is known as one of the major factors of cancer onset and progression [15, 16]. Earlier, the involvement of different pseudogenes in cancer development and progression has been disclosed by different studies [17-19]. The Pituitary tumor-transforming 3 pseudogene (PTTG3P), is a pseudogene having higher homology with its other family members including pituitary tumor-transforming 1 (PTTG1) and 2 (PTTG2) [20]. The PTTG3P gene is normally expressed in a wide variety of human tissues [21], however, in case of human cancers, along with its other family members (PTTG1 and PTTG2), this gene is reported to be specifically overexpressed in some human cancers such as pituitary adenoma [22], breast cancer [23], lung cancer, and gastric cancer [22]. Moreover, a study by *Huang et al.* revealed that overexpression of PTTG3P enhances the growth and development of hepatocellular carcinoma (HCC) by activating the PI3K/AKT signaling with the help of PTTG1 gene [24]. However, to the best of our knowledge, the role of the PTTG3P gene in the development, invasion, and metastasis of different other cancers is yet to be elucidated through pan-cancer analysis. Moreover, with the advances in high-throughput technology, the exploration of associations among the expression variations of oncogenes and different clinical factors has become essential. For this purpose, nowadays, the use of in silico techniques has gained a lot of attention. The benefit of in silico methods is that they can quickly predict the behavior of a large number of genes across disease samples

in a high-throughput mode. Another benefit of in silico approaches is that they can make predictions based on a protein-drug interaction to discover novel treatment options [25-28].

In this study, the TCGA project-based cancer datasets were used to conduct a pan-cancer analysis of PTTG3P gene for the first time. Furthermore, PTTG3P-associated some other important factors including survival status, methylation status, genetic changes, immune infiltration, and related cellular pathways were also explored to locate the underpin molecular mechanisms of ESCA and HNSC development using a series of publicly available databases, bioinformatics tools, and molecular experiments.

The expected outcomes of the present study may provide some useful pieces of information regarding correlations between PTTG3P expression, its prognostic values, and cancer development and progression. Our findings revealed PTTG3P as a potential biomarker for esophageal carcinoma (ESCA) and head and neck cancer (HNSC) patients. Moreover, we further observed that PTTG3P may also serve as a novel therapeutic target for cancer treatment. In a nutshell, via this study, we showed the potential regulatory mechanism of PTTG3P, identified its significance as a potential biomarker, and concluded that up-regulation of this gene is one of the major factors in regulating ESCA and HNSC development and progression.

### Methods

#### *The UALCAN*

The University of Alabama at Birmingham CANcer (UALCAN) (<http://ualcan.path.uab.edu>) is an efficient web portal that allows researchers to briefly analyze cancer-associated The Cancer Genome Atlas (TCGA) expression data [29]. We utilized this database for the pan-cancer differential expression, clinical variable-based expression analysis, and methylation analysis of PTTG3P in ESCA and HNSC patients. We utilized the "expression analysis" module of this database and "all cancer" data sets to analyze PTTG3P expression. Expression analysis of PTTG3P was analyzed in terms of pan-cancer different clinical variables. For statistics purpose, a student's t-test was employed

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**Table 1.** Information of ESCA and HNSC datasets used in the present study

Clinicopathological parameters-based classification of ESCA patients					
Sr. No	Parameter	Number of samples	Sum of ESCA samples	Sum of excluded samples due to missing details	Total number of samples undertaken analysis
1	Cancer stage distribution		184	29	155
	Stage 1	13			
	Stage 2	78			
	Stage 3	55			
	Stage 4	09			
2	Gender distribution			01	183
	Male	157			
	Female	26			
3	Nodal metastasis statuses based distribution			24	160
	N0	76			
	N1	69			
	N2	12			
	N3	03			
Clinicopathological parameters-based classification of ESCA patients					
Sr. No	Parameter	Sum of samples	Sum of ESCA samples	Sum of excluded samples due to missing details	Total number of samples undertaken analysis
1	Cancer stage wise distribution		184	29	155
	Stage 1	13			
	Stage 2	78			
	Stage 3	55			
	Stage 4	09			
2	Geographical distribution			20	164
	Caucasian	113			
	African-American	05			
	Asian	46			
3	Gender wise distribution			01	183
	Male	157			
	Female	26			

ESCA = Esophageal carcinoma and HNSC = Head and Neck squamous cell carcinoma.

in UALCAN and a value of  $p < 0.05$  was considered as statistically significant. Detail of the ESCA and HNSC datasets is provided in **Table 1**.

### *The Human Protein Atlas (HPA)*

The PTTG3P protein level in human normal and cancerous tissue was accessed through the Human Protein Atlas (HPA) database (<https://www.proteinatlas.org>) [30]. HPA database is an online biological research platform which is based on the TCGA database. In order to assess the expression of proteins in tissues and cells, including the localization and distribution of hundreds of proteins in distinct cancer tissues, HPA employs a number of combinatorial

approaches. A  $p$ -value  $< 0.05$  was considered as significant.

### *KM plotter based analysis*

The Kaplan Meier (KM) plotter (<https://kmplot.com/analysis/>) holds gene expression data (RNA-seq based) of more than 20 cancers. This tool helps the researchers in the validation and discovery of novel potential biomarkers [31]. With the aid of this tool in our study, we analyzed the correlation between PTTG3P transcriptomics expression and the overall survival (OS) of different cancer patients. In order to automatically construct the Kaplan-Meier survival maps, the genes to be examined are entered into the database and classified into

high and low groups based on their level of expression in the tumour samples. A *p*-value, 95% confidence interval (CI), and hazard ratio (HR) were determined and displayed.

### *The cBioportal*

cBioportal (<https://www.cbioportal.org/>) is an easy-to-use, publicly open-access platform which provides web-based resources for analyzing the TCGA cancer genomics data. It also facilitates researchers to obtain complete clinical and cancer genomic profiles of cancer patients [32]. In this study, alteration frequencies of PTTG3P were performed based on genomic mutations and DNA copy-number alterations in two (ESCA and HNSC) TCGA datasets.

### *PPI network and enrichment analysis*

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (<http://string-db.org>, version 11.0) is an authentic resource for analyzing functional network of the gene(s) of interest, which helps to understand the protein-protein interaction and underlying mechanisms of the genes [33]. In the present study, we used this source for constructing the protein-protein interaction (PPI) network of the PTTG3P enriched genes. This PPI network was then visualized by Cytoscape software [34]. Gene Ontology (GO) and Encyclopedia of Genes and Genomes (KEGG) analysis of the PTTG3P enriched genes was performed through DAVID [35]. GO analysis included three parts: biological process (BP), cell composition (CC), molecular function (MF), and *p*-value <0.05 was considered as significant.

### *PTTG3P and infiltrating level of CD8+ T cells*

Tumor IMMune Estimation Resource (TIMER, <https://cistrome.shinyapps.io/timer/>) database is an efficient and effective resource for analyzing tumor immune relatedness [36]. This database may precisely measure tumour purity and immune infiltration level and assess the relationship between invasion and clinical prognosis using RNA-seq expression profile data. We used this database for analyzing the correlation among mRNA expression of PTTG3P and CD8+ T immune cells level across different cancers. A *p*-value <0.05 was considered as significant.

### *PTTG3P gene-drug interactions*

The Comparative Toxicogenomics Database (CTD, <http://ctdbase.org/>) [37] is a cutting-edge online database that offered knowledge based on literature on the interactions between oncogene and different chemotherapeutic drugs. This database was searched in this study to obtain the PTTG3P associated drugs for gene-drug interaction network construction and analysis using Cytoscape software.

### *Real-world RNA-sequencing based in vitro validation of PTTG3P expression and promoter methylation*

Cell lines: One HNSC cell line (FaDu) and one normal human oral keratinocyte (HOK) cell line were purchased from the ATCC (American Type Culture Collection). The purchased cell lines were cultured in DMEM (HyClone), supplemented with 10% fetal bovine serum (FBS; TBD), 1% glutamine, and 1% penicillin-streptomycin in 5% CO<sub>2</sub> at 37°C.

Nucleic acid extraction: Total cell DNA from cell line samples was extracted using an organic method [38], while total RNA was extracted using the Trizol method [39]. We employed the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to assess the concentration and purity of the extracted DNA and RNA, ensuring that the A260/A280 ratio fell within the range of 1.8 to 2.0.

Library preparation for targeted bisulfite sequencing analysis: In brief, total DNA (1 µg) was fragmented into approximately 200-300 bp fragments using a Covarias sonication system (Covarias, Woburn, MA, USA). Following purification, the DNA fragments underwent repair and phosphorylation of blunt ends using a mixture of T4 DNA polymerase, Klenow Fragment, and T4 polynucleotide kinase. The repaired fragments were then 3' adenylated using Klenow Fragment (3'-5' exo-) and ligated with adapters containing 5'-methylcytosine instead of 5'-cytosine and index sequences using T4 DNA Ligase. The constructed libraries were quantified using a Qubit fluorometer with the Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and sent to Beijing Genomic Institute (BGI), China for targeted bisulfite sequencing. Following sequencing, the methylation data was normalized into beta values.

Library preparation for RNA sequencing analysis: RNA sequencing libraries were prepared using TruSeq RNA Access library kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's protocol.

After the completion of bisulfite-seq and RNA-sequencing analyses, methylation and gene expression values of PTTG3P were normalized using beta values and fragments per kilo base million reads (FPKM). The obtained beta values and FPKM values against PTTG3P in HNSC and normal oral keratinocyte (HOK) cell line cell were compared to identify differences in the promoter methylation and expression levels.

### Results

#### *PTTG3P aberrantly expressed in different types of human cancers*

The aim of our study is to comprehensively explore the oncogenic effects of the human PTTG3P gene via pan-cancer analysis. We analyzed the transcriptional level of PTTG3P across tissue samples of various cancers and their paired normal controls using UALCAN platform. The dysregulated transcriptional level of PTTG3P was identified in all the analyzed 24 different types of human cancers in the UALCAN database. According to our results, as shown in **Figure 1**, the transcriptional level of PTTG3P was significantly lowered in one type of cancer samples (Thyroid Cancer, THCA) relative to controls, while significantly higher in the other remaining 23 types of cancer samples relative to controls (**Figure 1**).

#### *PTTG3P has prognostic potential in cancers*

In KM plotter, all the cancer samples were divided into two groups, i.e., high and low-expression groups with respect to PTTG3P expression, and then, these groups were utilized to exploit the prognostic values of PTTG3P. We assessed the relationship between the mRNA expression of individual PTTG3P and the survival of different patients with cancer. As shown in **Figure 2**, the survival curves demonstrated that overexpression of PTTG3P (hazard ratio [HR] = 3.94, 95% confidence interval [CI]: 1.61 to 9.66, P = 0.0014), (hazard ratio [HR] = 1.38, 95% confidence interval [CI]: 1 to 1.9, P = 0.049) was significantly correlated with the shorter overall survival (OS) of the ESCA and HNSC patients, respectively. Collectively, these

data suggested that PTTG3P might have a significant contribution in the development and progression of ESCA and HNSC, therefore, the next part of our study will mainly focus on the unique role of PTTG3P in these two cancers.

#### *PTTG3P expression and clinical indicators of ESCA and HNSC*

Given the significant role of overexpressed PTTG3P in the development and progression of ESCA and HNSC, we utilized the UALCAN web resource to document the correlations among PTTG3P expression and different clinical indicators of ESCA and HNSC patients. As shown in **Figure 3**, the results of the present study highlighted the significant (P<0.05) higher expression of PTTG3 across ESCA and HNSC patients stratified by individual cancer stage, patient's race, patient's gender, and patient's age groups (**Figure 3**). Based on individual cancer stage, PTTG3 expression was higher in stage 2 and stage 3 ESCA and HNSC patients as compared to stage 1 and 4 patients (**Figure 3**). Concerning patient's race, ESCA and HNSC patients of Caucasian population exhibited the higher expression of PTTG3P relative to African-American and Asian populations (**Figure 3**). As far patient's gender, PTTG3P expression was higher in male ESCA and HNSC patients as compared to female patients (**Figure 3**). Based on patient's age groups, PTTG3P has shown high expression in 41-60 Yrs ESCA and HNSC samples relative to other age groups (**Figure 3**).

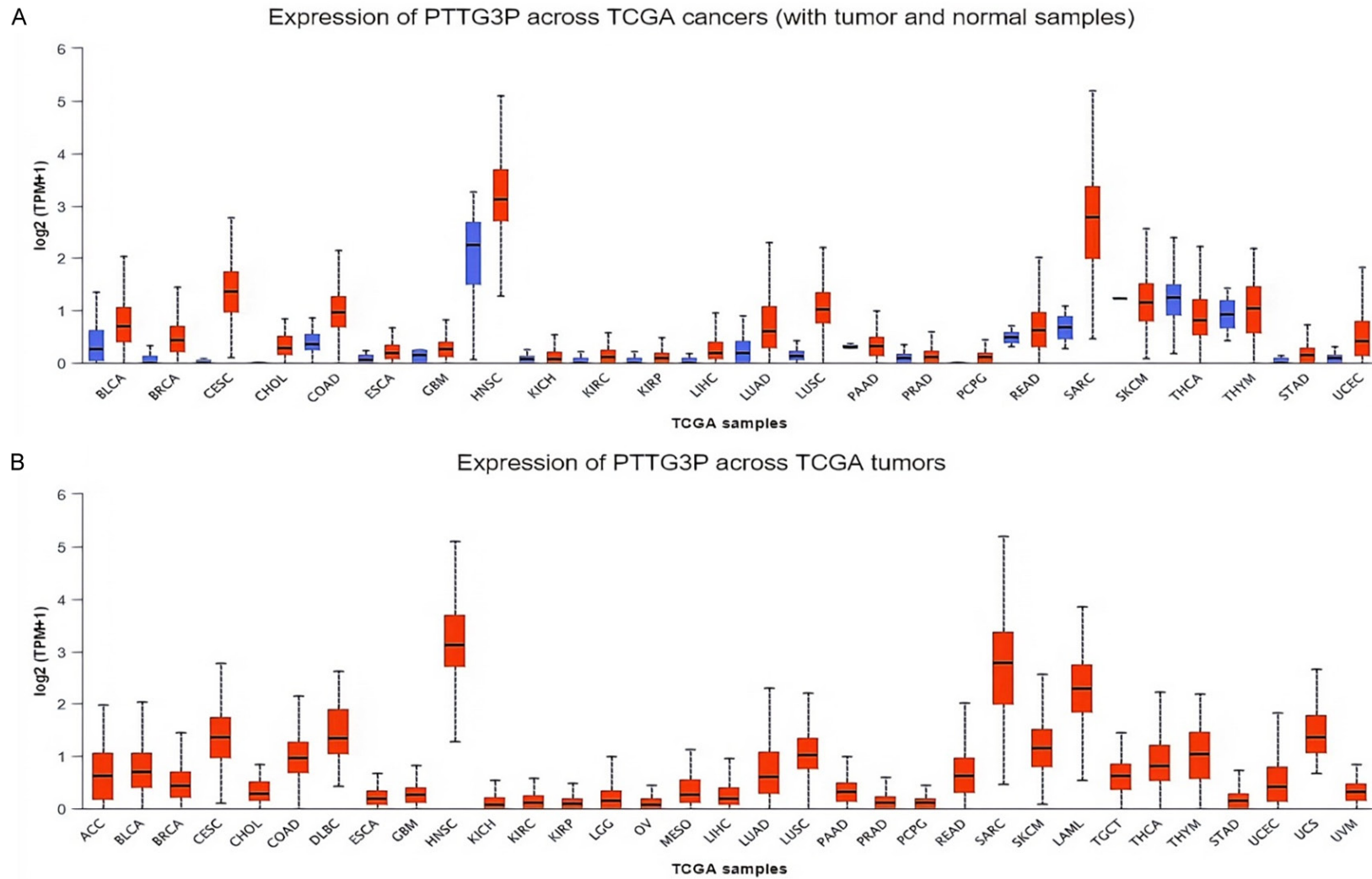
#### *Elevated translational level of PTTG3P*

After documenting PTTG3P mRNA expression and its correlation with cancer patients' prognosis, we then documented the PTTG3P protein expression using the HPA database. The antibody used for staining of ESCA and normal samples was HPA045034, while in case of HNSC and normal samples, the antibody was HPA008890. As a result, we found that PTTG3P proteins' intensity was not detected in normal esophageal and head and neck tissues. However, relative to normal controls, a PTTG3P protein was obviously found to be significantly overexpressed (high) in esophageal and head and neck cancerous tissues (**Figure 4**).

#### *PTTG3P promoter methylation analysis*

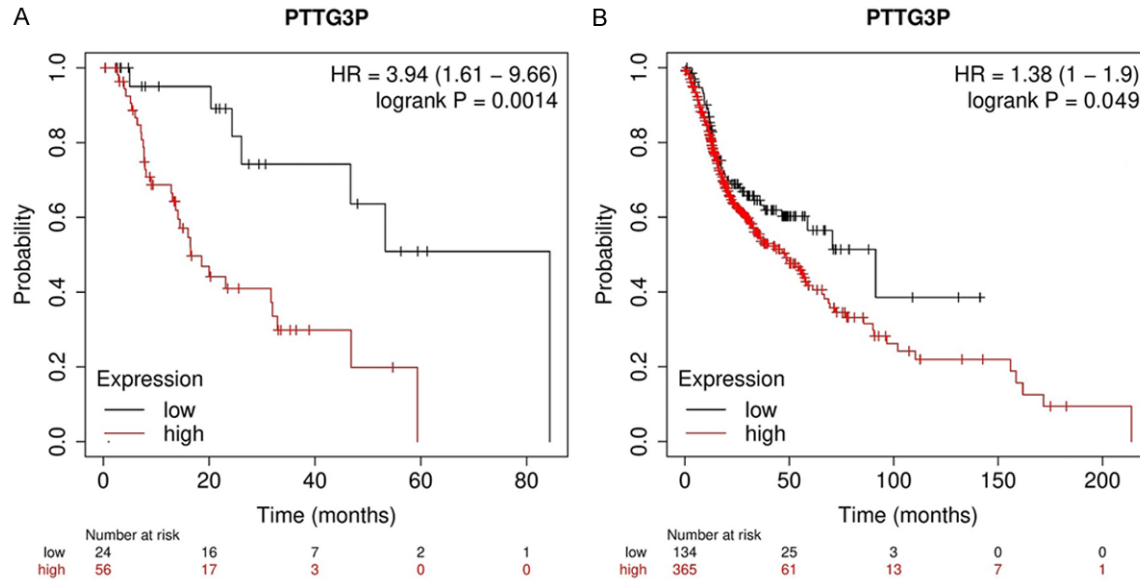
Genetic modulation is known as one of the major factors for dysregulating the mRNA

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**Figure 1.** Expression profiling of PTTG3P across different cancers documented by UALCAN. (A) Expression profiling of PTTG3P gene in cancer tissue samples paired with their normal corresponding control samples, and (B) Expression profiling of PTTG3P in normal control tissues samples. A  $p$ -value  $< 0.05$  was considered as significant. PTTG3P = Pituitary tumor-transforming 3 pseudogene, UALCAN = The University of Alabama at Birmingham CANcer.

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**Figure 2.** KM plotter based identified prognostic values of PTTG3P. (A) A higher expression of PTTG3P was found to be associated with the poor OS in ESCA patients, and (B) A higher expression of PTTG3P was found to be associated with the poor OS in HNSC patients. A  $p$ -value  $<0.05$  was considered as significant. PTTG3P = Pituitary tumor-transforming 3 pseudogene, OS = Overall survival, ESCA = Esophageal carcinoma, HNSC = Head and neck cancer.

expression level of any gene. By conducting UALCAN analysis, we explored if the expression of PTTG3P mRNA was regulated by promoter DNA methylation. Results revealed that the promoter methylation level of PTTG3P was significantly ( $P < 0.05$ ) lower in ESCA, while significantly ( $P < 0.05$ ) higher in HNSC samples in comparison to normal controls (**Figure 5**). Hence, we speculated that PTTG3P up-regulation in ESCA maybe because of PTTG3P promoter hypomethylation, however, the PTTG3P promoter methylation scenario in HNSC challenges the classical view of hypermethylation where it is always correlated with the down-regulation [40]. These results may owe to the fact that there was not a significantly negative correlation between PTTG3P mRNA expression and DNA promoter methylation in HNSC samples.

### Genomics alteration analysis

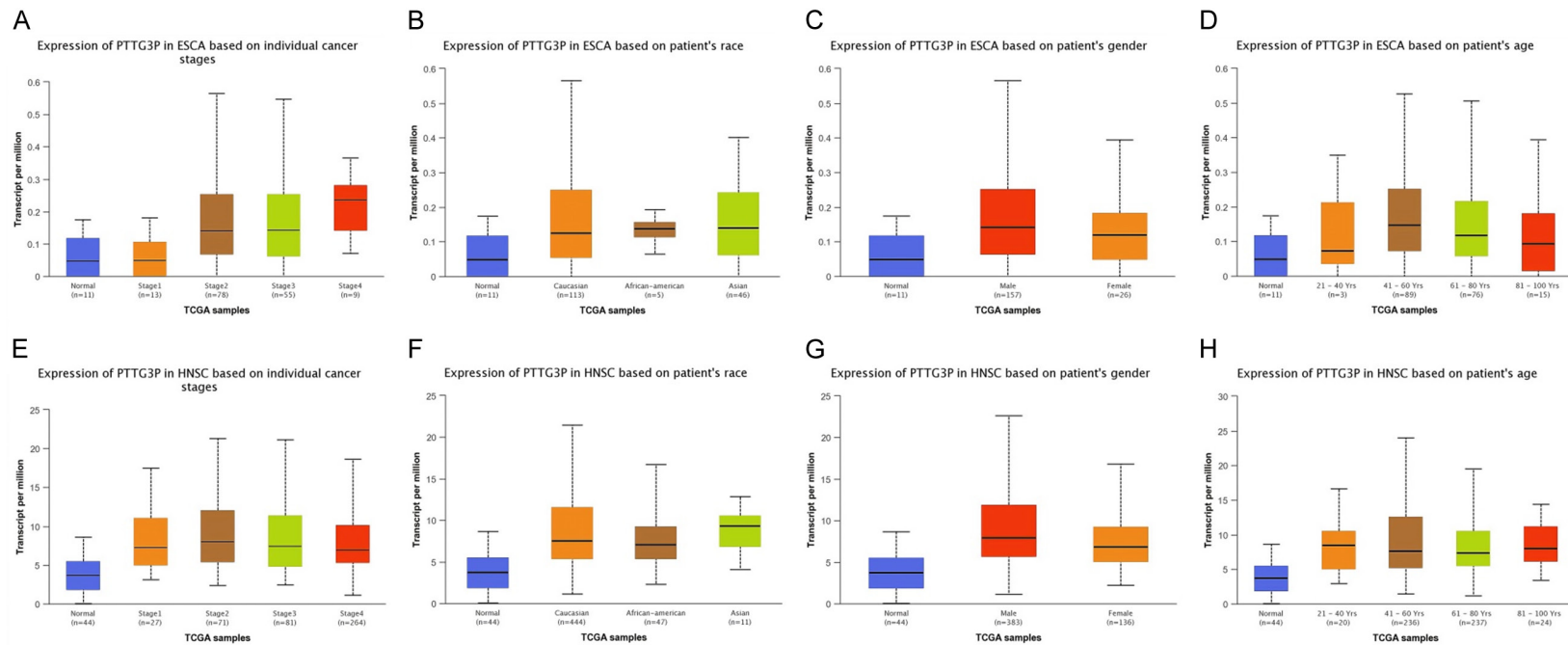
Via TCGA projects, we found the genomics alteration status of the PTTG3 gene across ESCA and HNSC datasets. As highlighted in **Figure 6**, we used Esophageal Carcinoma (TCGA, Nature 2017 consisting of 559 cancerous samples), and Head and Neck Squamous Cell Carcinoma (TCGA, Pancancer Atlas con-

sisting of 523 cancerous samples) datasets for this purpose, and our results highlighted that ESCA samples in the selected dataset have a 5% genomics alteration frequency, while HNSC samples in the selected dataset have a 3% genomics alteration frequency. It is important to note that all genetically altered ESCA and HNSC samples showed deep amplification genomic alteration, which may be one of the reasons for PTTG3P up-regulation (**Figure 6**).

### PPI network and pathway analysis of PTTG3P

Together, the STRING database and Cytoscape software screened a complex network of a total of 11 PTTG3P-associated genes. All of these genes are validated through experimental data and the interactions between PTTG3P and these 11 genes are shown in **Figure 7A**. Next, to understand the molecular mechanism of PTTG3P oncogenic role in association with these 11 genes, we conducted the GO and KEGG enrichment analysis of these genes using the DAVID tool. As shown in **Figure 7B**, our results revealed the notable involvement of these genes in the dysregulation of different diverse pathways including "Oocyte meiosis", "Cell cycle", "Ubiquitin mediated proteolysis", and "Progesterone-mediated oocyte

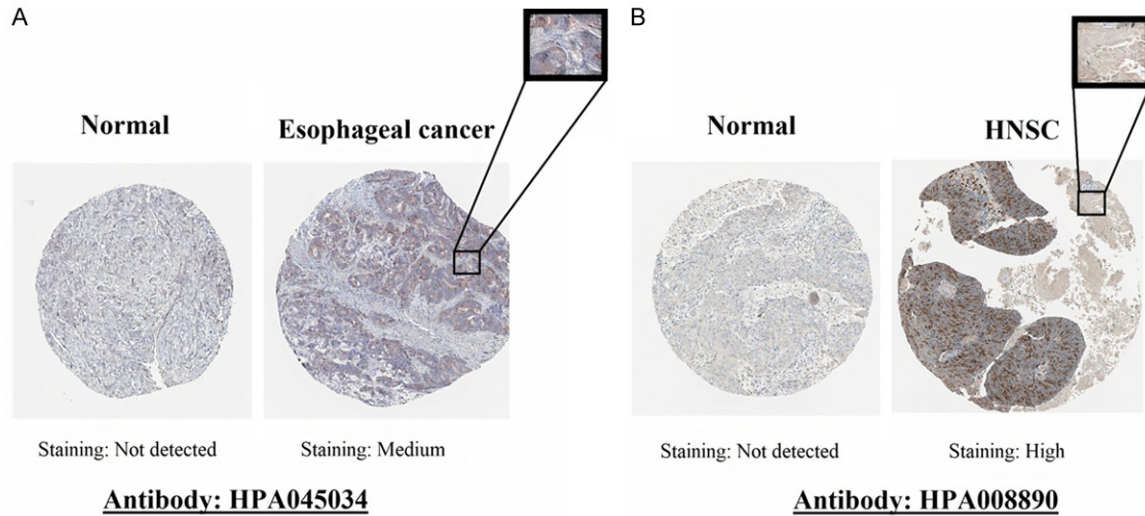
## PTTG3P role in cancer



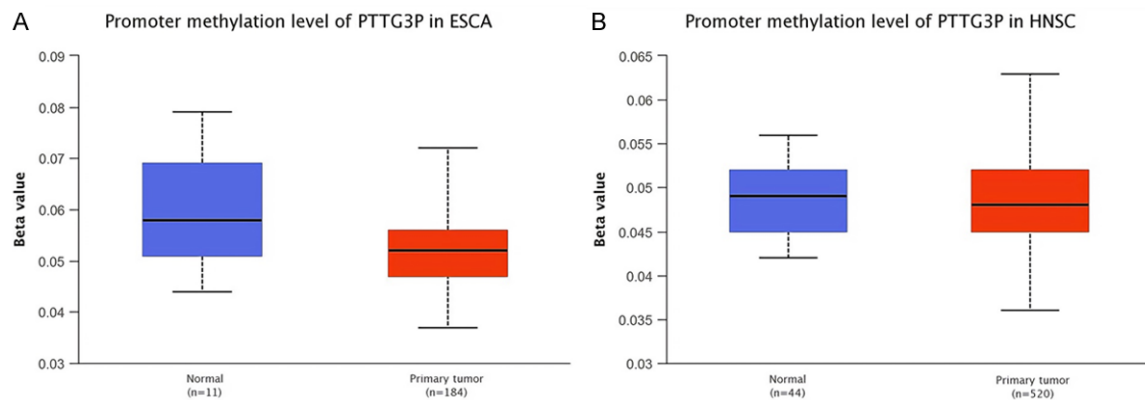
**Figure 3.** Different clinical variables-wise expression profiling of PTTG3P in ESCA and HNSC. (A) Individual cancer stage-wise PTTG3P expression profiling in ESCA, (B) Patient's race-wise PTTG3P expression profiling in ESCA, (C) Patient's gender-wise PTTG3P expression profiling in ESCA, (D) Patient's age-wise PTTG3P expression profiling in ESCA, (E) Individual cancer stage-wise PTTG3P expression profiling in HNSC, (F) Patient's race-wise PTTG3P expression profiling in HNSC, (G) Patient's gender-wise PTTG3P expression profiling in HNSC, and (H) Patient's age-wise PTTG3P expression profiling in HNSC. A  $p$ -value  $<0.05$  was considered as significant. PTTG3P = Pituitary tumor-transforming 3 pseudogene, ESCA = Esophageal carcinoma, HNSC = Head and neck cancer.



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**Figure 4.** Immunohistochemistry images of PTTG3P protein expression in esophageal and head and neck cancers along with normal controls taken from Human Protein Atlas (HPA) database ( $\times 200$ ). (A) Immunohistochemistry images of PTTG3P protein expression in esophageal cancer tissue paired with control, and (B) Immunohistochemistry images of PTTG3P protein expression in head and neck cancer tissue paired with control. PTTG3P = Pituitary tumor-transforming 3 pseudogene.



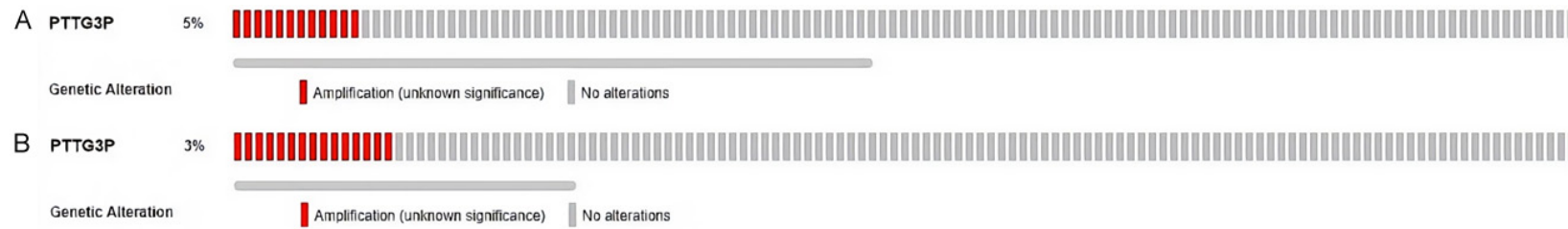
**Figure 5.** Box plot showing PTTG3P promoter methylation level in ESCA and KIRC tissues samples and normal control tissue samples. (A) Boxplots presenting PTTG3P promoter methylation in ESCA tissue samples paired with controls, and (B) Boxplots presenting PTTG3P promoter methylation in HNSC tissue samples paired with controls. A  $p$ -value  $< 0.05$  was considered as significant. PTTG3P = Pituitary tumor-transforming 3 pseudogene, ESCA = Esophageal carcinoma, HNSC = Head and neck cancer.

maturation" (Table 2). Moreover, via GO analysis, it was also noted that PTTG3P enriched genes were associated with a variety of GO terms (Figure 7C-E). For example, with Anaphase-promoting complex-dependent catabolic process, Reg. of mitotic sister chromatid separation, Mitotic nuclear division etc., BP terms (Figure 7C), Chromosome passenger complex, Anaphase-promoting complex, Spindle pole centrosome CC terms (Figure 7D), and Histone serine kinase activity, Anaphase promoting complex binding and Histone kinase activity etc., MF terms (Figure 7E).

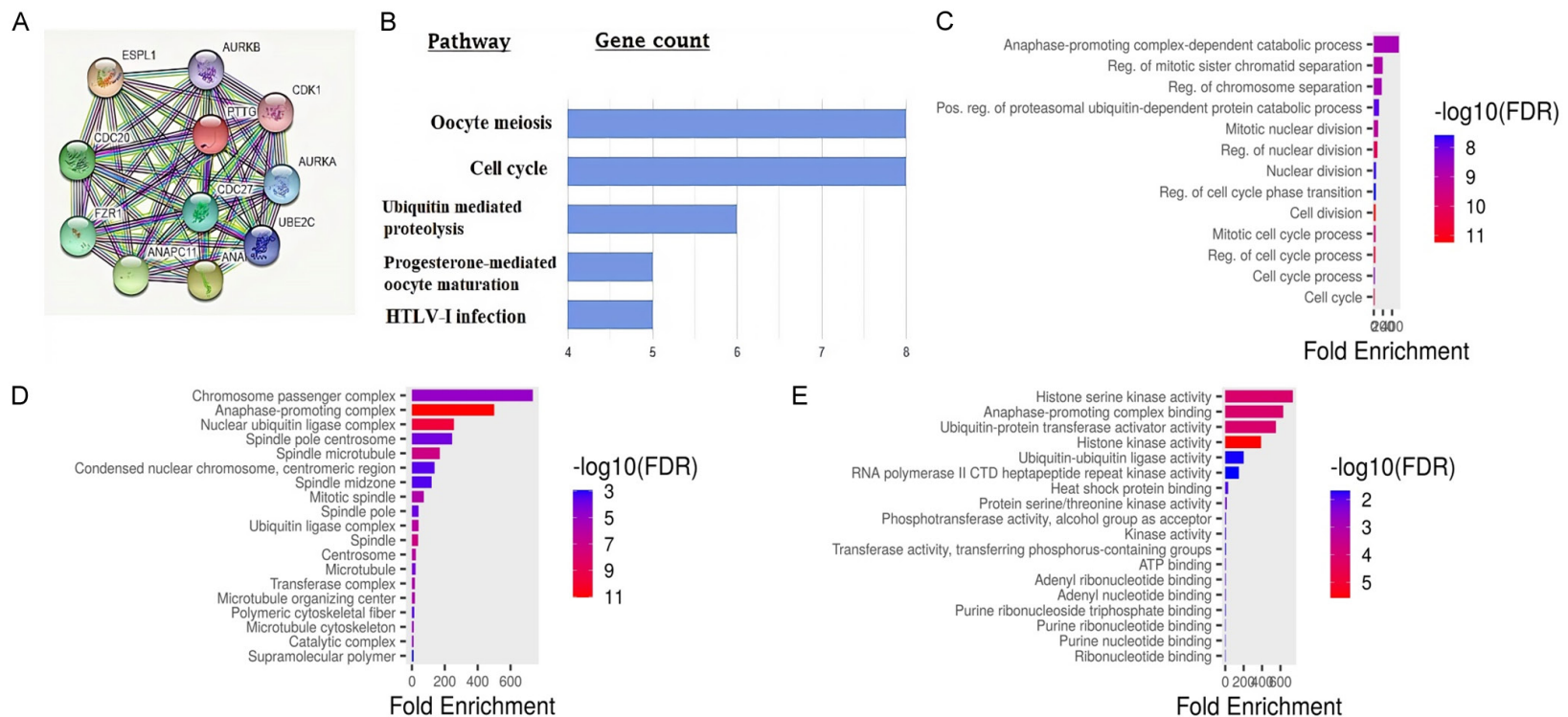
### *CD8+ T immune infiltrates in correlation with PTTG3P*

The tumor-infiltrating immune cells, especially the CD8+ T cells were significantly associated with the development, growth, progression, and metastasis of cancer cells to distant parts of the body as a key regulator of the tumor microenvironment. The working of these immune cells has earlier been reported to be influenced by the activity of tumor-associated fibroblasts in the tumor microenvironment. We discovered significant ( $P < 0.05$ ) positive corre-

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**Figure 6.** PTTG3P-associated genomic mutations and CNVs in ESCA and HNSC TCGA datasets retrieved from cBioPortal. (A) PTTG3P-associated genomic mutations and CNVs in ESCA, and (B) PTTG3P-associated genomic mutations and CNVs in HNSC. PTTG3P = Pituitary tumor-transforming 3 pseudogene, CNV = Copy number variation, TCGA = The Cancer Genome Atlas, ESCA = Esophageal carcinoma, HNSC = Head and neck cancer.

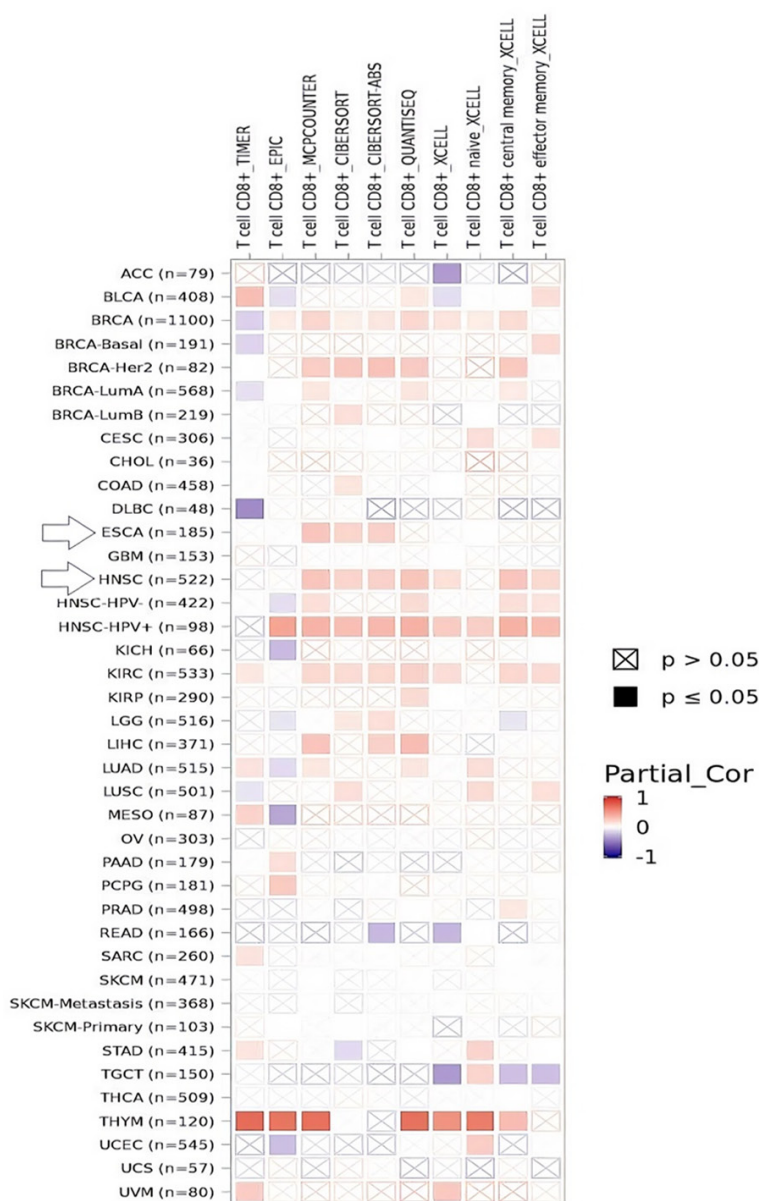


**Figure 7.** PPI network construction and pathway enrichment analysis. (A) A constructed PPI network of PTTG3P-associated genes, (B) A heat map highlighting PTTG3P-associated genes KEGG terms, (C) A heat map highlighting PTTG3P-associated genes BP terms, (D) A heat map highlighting PTTG3P-associated genes CC terms, and (E) A heat map highlighting PTTG3P-associated genes MF terms. A  $p$ -value  $<0.05$  was considered as significant. FDR = False discovery rate.

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**Table 2.** KEGG pathway items of PTTG3P-associated genes

ID	Pathway	Gene count	P-value	Gene ID
04114	Oocyte meiosis	8	<0.05	CDC20, PTTG3, ESPL1, CDC27, CDK1, AURKA, ANAPC11, ANAPC2
04110	Cell cycle	8	<0.05	CDC20, FZR1, PTTG3, ESPL1, CDC27, CDK1, ANAPC11, ANAPC2
04120	Ubiquitin mediated proteolysis	6	<0.05	CDC20, FZR1, UBE2C, CDC27, ANAPC11, ANAPC2
04914	Progesterone-mediated oocyte maturation	5	<0.05	FZR1, CDC27, CDK1, ANAPC11, ANAPC2
05166	HTLV-I infection	5	<0.05	CDC20, PTTG3, CDC27, ANAPC11, ANAPC2



**Figure 8.** Association between PTTG3P expression and CD8+ T immune cell level in ESCA and HNSC via TIMER database. A p-value <0.05 was considered as significant. PTTG3P = Pituitary tumor-transforming 3 pseudogene, ESCA = Esophageal carcinoma, HNSC = Head and neck cancer.

lations between the mRNA expression of PTTG3P and CD8+ T immune cells level in ESCA and HNSC using the TIMER database (**Figure 8**). In view of our results, we speculated that infiltration of CD8+ T immune cells in ESCA and HNSC tumors might be associated with PTTG3P expression and thus may affect the cancer patient's prognosis.

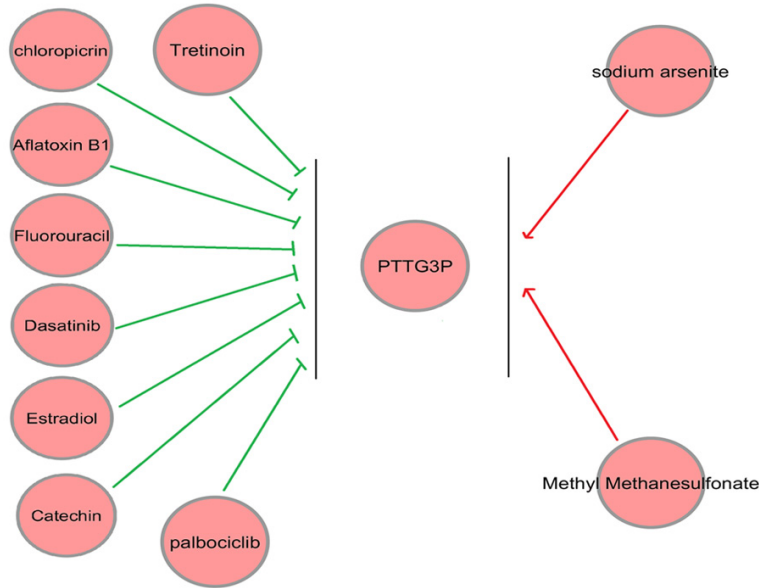
### *PTTG3P gene-drug interaction network analysis*

Taking advantage of the CTD database, we aimed to screen potential therapeutic compounds that could affect the mRNA expression of the PTTG3P gene in ESCA and HNSC patients. In view of the analysis results, it was observed that PTTG3P expression can be regulated by a variety of drugs. For example, methyl methanesulfonate and sodium arsenite can elevate while cloropicrin and tretinoin can reduce PTTG3P expression (**Figure 9**).

### *Real world experimental in vitro validation of PTTG3P expression and promoter methylation*

In the present investigation, the validation of PTTG3P gene expression was performed using RNA-seq data from one

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**Figure 9.** A gene-drug interaction network highlighting PTTG3P-associated chemotherapeutic drugs. Red arrows: chemotherapeutic drugs that can increase PTTG3P expression, green arrows: chemotherapeutic drugs that can decrease PTTG3P expression, numbers of arrows: supported numbers of studies in the literature. PTTG3P = Pituitary tumor-transforming 3 pseudogene.

HNSC (FaDu) cell line and one normal human oral keratinocyte (HOK) cell line. The quantification of PTTG3P gene expression utilized FPKM, a widely accepted quantitative measure in RNA-seq analysis. The results depicted in **Figure 10** illustrated the presence of PTTG3P gene expression in both cell lines, with notably higher FPKM values observed in the HNSC cell line (FaDu) in comparison to the normal cell line (HOK) (**Figure 10A**).

Additionally, employing bisulfite-seq analysis, disparities in the methylation status of the PTTG3P promoter were identified. Specifically, beta values associated with PTTG3P promoter methylation were found to be notably lower in the FaDu cell line when contrasted with the normal control cell line (HOK), suggesting a state of hypomethylation for this gene (**Figure 10B**).

### Discussion

At present, the state of knowledge regarding the diagnostic and prognostic potential of PTTG3P in human cancers is very limited. In our study, we aimed to highlight the PTTG3P potential as a novel potential diagnostic and prognostic biomarker of cancers using a series

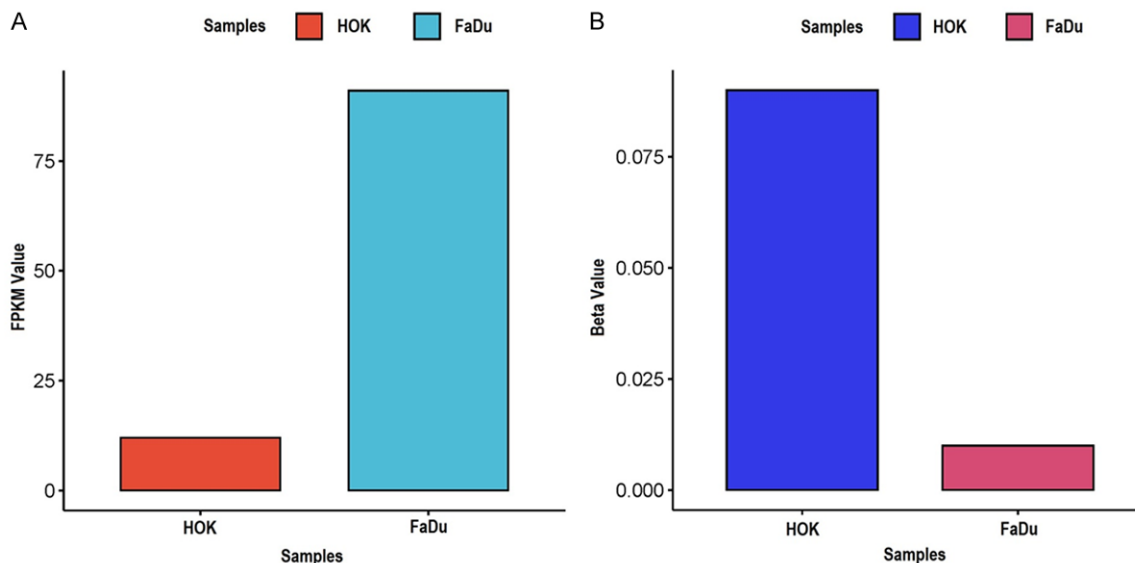
of in silico and molecular analyses.

Pseudogenes were considered as junk genes for a long time [41, 42]. However, a recent growing body of evidence has suggested many key roles of pseudogenes in pathological processes, for example, the abnormal expression pattern of pseudogenes is closely related with human cancers [43, 44]. Many cancer-related pseudogenes have been discovered so far, such as PTENP1 [45], POU5F1B [17], HMGA1P6, HMGA1P7 [46], FOXO3P [47], and INTS6P1 [48]. The PTTG3P is also a recently discovered novel pseudogene, which has closely been related to the human cancer development and progression, such as hepatocellular carcinoma

and gastric cancer [24, 49]. However, its expression dysregulation in different other human cancers and underlying mechanisms, remain largely unknown.

In this study, we analyzed the mRNA expression level variations in PTTG3P in pan-cancer and the paired control tissue samples via UALCAN and HPA databases. The PTTG3P expression was explored to be significantly higher across tumor samples relative to normal tissues in various cancer types (**Figure 1**). Differences in the utilized data collection approaches and analytical techniques may be one of the factors attributed to the heterogeneity of PTTG3P expression across different cancer types. However, through KM plotter analysis, we observed that the elevated expression of PTTG3P in ESCA and HNSC patients was significantly ( $P < 0.05$ ) correlated with the worst prognosis. These results suggest that PTTG3P is an oncogene in ESCA and HNSC and its expression elevation play a significant role in the development and progression of ESCA and HNSC. As for another important finding of this study, the PTTG3P was also found significantly ( $P < 0.05$ ) overexpressed in ESCA and HNSC patients of different clinicopathological indica-

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**Figure 10.** HOK and FaDu cell lines-based in vitro expression and promoter methylation levels validation of PTTG3P gene. (A) RNA-seq based expression validation of PTTG3P gene in FaDu and HOK cell lines, and (B) Bisulfite-seq based promoter methylation validation of PTTG3P gene in FaDu and HOK cell lines. PTTG3P = Pituitary tumor-transforming 3 pseudogene.

tors including individual cancer stages, patient's race, patient's gender and patient's age groups.

In the current study, a total of two TCGA datasets (one ESCA and one HNSC) were analyzed to document the genomic alterations in PTTG3P genes across ESCA and HNSC samples via cBioPortal. Our results highlighted that ESCA samples in the selected dataset had 5% genomics alteration frequency, while HNSC samples in the selected dataset had 3% genomics alteration frequency in PTTG3P gene. Deep amplification genetic abnormality at gene level is one of the core reasons for higher level of the expressions [50, 51]. Earlier, it is noted that gene amplification causes the up-regulation of certain important genes across different cancers. For example, across multiple myeloma, gene amplification genomic was explored to up-regulate the expressions of DUSP8, PRG2, ERMN, RNASE2, and BMF genes [52]. It is important to note that all genetically altered ESCA and HNSC samples in our study showed deep amplification genomic alteration in PTTG3P gene, which may be one of the reasons of the PTTG3P up-regulation. In addition to genomic alteration analysis, we further conducted the promoter methylation analysis of PTTG3P gene across ESCA and HNSC samples.

Based on the analysis results, PTTG3P promoter methylation revealed an expected negative correlation with its mRNA expression across ESCA samples while an unexpected positive correlation with its mRNA expression in HNSC samples, which challenges the classical view where hypermethylation is always correlated with mRNA down-regulation [40].

As for ESCA and HNSC biomarkers, expression changes in different genes have been earlier noted as potential candidate signatures. For example, expression changes in EGFR, CCND1, DGCR8, ErbB2, POM121, UPF3B, IGF1R, TAF9, BCAP31, BUB1, TOP2A, DLGAP5, CENPF, TPX2, ASPM, AURKA, UBE2C, CDC20, and NEK2 genes were considered as the potential candidate signatures for diagnosis and predicting the prognosis ESCA patients [53-55]. Similarly, expression changes in GPR18, MYL2, CNR2, VCAN, ULBP2, STC2, S100A8, S100A9, LAMB1, IL1RN, ANXA1, PPL, KRT4, TGM3, SCEL, PSCA, CSTA, FSTL1, TEX101, SDC2, IGFBP7, RSPH4A, ACTN2, and TTN genes were considered as the potential candidate signatures for diagnosis and predicting the prognosis HNSC patients [56-59]. However, best to our knowledge, none of these or any other biomarker has been generalized so far in the ESCA and HNSC patients of different clinicopathological vari-

ables. In the present study, we revealed the significant ( $P < 0.05$ ) up-regulation of PTTG3P expression in ESCA and HNSC patients with different clinical variables including different cancer stages, patients' races, patients' genders, and patients body weights as compared to the normal controls. We have also shown that PTTG3P overexpression is significantly ( $P < 0.05$ ) associated with the worst OS of the ESCA and HNSC patients. Therefore, in view of this, PTTG3P up-regulation was suggested as a novel diagnostic and prognostic biomarker of ESCA and HNSC patients regardless of clinical variables.

The microenvironment of a tumor consists of a wide variety of cells and among these cells, infiltrating immune cells account constitute the major proportion [60]. On the other side of the coin, unlike the typical concept about immune cells as the important constituent of the anticancer therapy, the immune infiltration across tumor microenvironment shows the underpinning strategy of cancerous cells to avoid getting killed. Moreover, among all types of immune cells present in the tumor microenvironment, most of the immune cells especially CD8+ T cells along with important dysregulated genes were reported to be involved in development and regulation of cancer [61]. For example, according to recent research, the dysregulation of YY1 was found to be abnormally linked with a variety of immune cells infiltrates across various cancers [52]. The CD8+ T immune cells migrate from bloodstream to cancer tissue and perform various important tasks here in immune regulation [62, 63]. To understand the associations between PTTG3P expression and CD8+ T immune cells infiltration in our study, we detected the infiltration level of these cells in ESCA and HNSC samples. Our results showed significant positive correlations between PTTG3P expression and CD8+ T immune cells in ESCA and HNSC patients. Therefore, we speculate that PTTG3P may be a major immunomodulatory target in ESCA and HNSC progression. However, in this regard, the PTTG3P potential needs to be explored further.

Next, to explore the underlying molecular pathways of the PTTG3P gene in the development and progression of ESCA and HNSC, we used STRING and DAVID in this research. For this purpose, the PTTG3P-associated genes were obtained from STRING and subjected to analy-

sis via DAVID. Results of the analysis revealed the enrichment of PTTG3P genes in different important signaling pathways including "Oocyte meiosis", "Cell cycle", "Ubiquitin mediated proteolysis", and "Progesterone-mediated oocyte maturation". Additionally, we have also identified a few potential drugs that could be useful in the treatment of ESCA and HNSC by regulating PTTG3P expression.

### Conclusion

In summary, we performed extensive data mining and conducted a series of bioinformatics and molecular analyses to find the cancer-driving role of PTTG3P. We found that PTTG3P was highly expressed in ESCA and HNSC patients and had an adverse effect on the survival outcomes of these patients. PTTG3P-associated genes were mainly involved in various important GO and KEGG terms, therefore playing an essential role in the development and progression of ESCA and HNSC. In the near future, the present work may be used as reference data for newly conducted studies to further explore the oncogenic roles of PTTG3P in ESCA and HNSC patients using *in vivo* and *in vitro* experiments. Findings of the current study were mainly based on bioinformatics analyses. Validating using PTTG3P in-house clinical samples could greatly strengthen our findings.

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### Disclosure of conflict of interest

None.

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