ENST00000489707.5 Is a Preferred Alternative Splicing Variant of PTK7 in Adrenocortical Cancer and Shows Potential Prognostic Value

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Background: This study aimed to explore the transcript preference of PTK7 in adrenocortical cancer (ACC), the prognostic value, and the potential underlying genetic alterations.

Material/Methods: Data from the Cancer Genome Atlas-Adrenocortical Cancer (TCGA-ACC) and the Genotype-Tissue Expression (GTEx)-normal adrenal gland were used for analysis.

Results: A non-canonical alternative transcript, ENST00000489707.5, which only encodes an extracellular immunoglobulin (Ig)-like domain and an intracellular kinase domain, is the dominant isoform of PTK7 in both ACC and normal adrenal gland. Its expression percentage was significantly higher in ACC than in normal adrenal gland. ACC tissues showed preferred expression of this transcript compared with other cancers with known PTK7 expression. Prognostic analysis showed that ENST00000489707.5 had independent prognostic value in progression-free survival (PFS) (HR: 1.227, 95% CI: 1.077–1.398, p = 0.002) and disease-specific survival (DSS) (HR: 1.419, 95% CI: 1.154–1.745, p = 0.001) after adjustment of other risk factors. cg20819617 methylation was negatively correlated with both PTK7 and ENST00000489707.5 expression.

Conclusions: ENST00000489707.5 is a preferred alternative splicing product of PTK7, with a significantly increased proportion in ACC compared with other cancers. Its expression shows potential prognostic value in terms of PFS and DSS in ACC patients. The methylation status of cg20819617 might play a critical role in modulating PTK7 transcription and ENST00000489707.5 expression.

MeSH Keywords: Adrenal Cortex Neoplasms • Alternative Splicing • Prognosis • Receptor Protein-Tyrosine Kinases

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Background

Tyrosine-protein kinase-like 7 (PTK7) is a receptor tyrosine kinase that is encoded by the PTK7 gene in the human genome. This kinase is also called colon carcinoma kinase 4 (CCK4). This protein does not have detectable catalytic tyrosine kinase activity and thus is considered as a pseudokinase [1]. PTK7 gene has 20 exons, which span approximately 85 kb in the human genome. The full-length PTK7 protein contains 7 extracellular immunoglobulin-like (Ig) domains (encoded by exons 2 to 13), a transmembrane region (encoded by the 5’-half of exon 14), a juxtamembrane region (encoded by 3’-half of exon 14 and 5’-half of exon 15), and a catalytically inert cytoplasmic tyrosine kinase domain (encoded by the 3’-half of exon 15 and exons 16 to 20) [2].

The extracellular immunoglobulin-like (Ig)-like domains of PTK7 proteins are required for WNT-binding [3]. Previous studies showed that PTK7 acts as a critical regulator of both canonical and non-canonical Wnt-signaling via selectively interacting with diffident molecules such as Wnt3a, Wnt8 [4], Wnt5a [3], LRP6 [5], and RACK1 [6] in different tissue environments. PTK7 can also interact with VEGFR1 [7] and Plexin A1 [8]. Via these pathways, PTK7 modulates a series of cellular processes such as polarity, angiogenesis, migration, and adhesion.

PTK7 upregulation was observed in multiple cancers, such as colorectal cancer [9], triple-negative breast cancer [10], lung adenocarcinoma [11], and cervical cancer [12]. In addition, its expression might be a high potential prognostic marker in lung adenocarcinoma [11], prostate cancer [13], and gastric cancer [14]. As a gene with 20 exons, PTK7 is subjected to alternative splicing after transcription, which generates multiple transcript isoforms encoding proteins with different lengths and structures [2]. The full-length PTK7 protein can be proteolyzed by MT1-MMP to generate an N-terminal, soluble PTK7 fragment (sPTK7) [15]. The C-terminal membrane PTK7 fragment can be further proteolyzed by disintegrin and metalloprotease 17 (ADAM17) and γ-secretase to release the intracellular domain of PTK7 [16]. These protein fragments have significant functional differences [17]. Therefore, the variants contribute to the functional diversity of PTK7 in different tissues and the expression of certain variants.

In this study, we aimed to explore the dysregulated PTK7 transcripts in adrenocortical cancer (ACC), as well as to assess the prognostic value and potential underlying genetic alterations, using data from the Cancer Genome Atlas-Adrenocortical Cancer (TCGA-ACC) and the Genotype-Tissue Expression (GTEx)-normal adrenal gland.

Material and Methods

Data acquisition from the Cancer Genome Atlas-Adrenocortical Cancer (TCGA-ACC)

The level-3 data in TCGA-ACC used in this study were extracted by using the UCSC Xena browser (https://xenabrowser.net/) [18]. A total of 92 ACC cases were included in this study. Among these 92 cases, 77 had progression-free survival (PFS) data and 75 had disease-specific survival (DSS) data, RNA-seq data of gene/transcript expression, and DNA methylation status. Gene expression profile was acquired using the Illumina HiSeq 2000 RNA Sequencing platform from the University of North Carolina TCGA Genome Characterization Center. Gene-level transcription is estimated by expert statisticians of the center using log2 (RSEM+1) transformation, in which RSEM refers to RNA-Seq by Expectation-Maximization. Gene transcript expression was calculated by Log, Transcript per Million (TPM). The β value of each CpG site was calculated for methylation estimation.

Briefly, the following data were collected: age at initial diagnosis, sex, pathological stages, nuclear grade, the presence of residual tumor, sinusoid invasion, Weiss venous invasion, invasion of tumor capsule, PFS time, and DSS time.

PTK7 transcript expression in the normal adrenal gland

PTK7 transcript expression data in normal adrenal glands were obtained from the Genotype-Tissue Expression (GTEx) database, which is a project to determine tissue-specific gene expression in normal human tissues [19,20], with access via the UCSC Xena browser. The expression and percentage of each transcript in normal adrenal glands was compared with the data in TCGA-ACC.

Statistical analysis

Data analysis was performed using the SPSS 25.0 software package (SPSS, Inc., Chicago, IL, USA), together with GraphPad Prism 8.04 (GraphPad Inc., La Jolla, CA, USA). Comparison between 2 groups was performed using an unpair t test with Welch’s correction. Kaplan-Meier (K-M) survival curves were generated to compare the survival difference between patients with high and low ENST00000489707.5 expression. The log-rank test was performed to test the statistical difference between the curves. The prognostic value of ENST00000489707.5 expression as a continuous variable was assessed using univariate and multivariate Cox regression models. Pearson’s correlation analysis was performed for correlation estimation. p<0.05 was considered statistically significant.
Results

**ENST00000489707.5 is the dominant isoform of PTK7 transcripts, with significantly increased proportion in ACC than in normal adrenal glands**

Using gene transcript data from both TCGA and GTEx, we compared the expression and proportion of **PTK7** transcripts in ACC and normal adrenal glands. Results showed that **PTK7** has complex alternative transcripts in both tumor and normal tissues, among which ENST00000489707.5 has the strongest expression (Figure 1A, 1B). This transcript contains 8 exons that encode a small protein with 365 amino acids. This protein variant contains an extracellular Ig domain and a kinase domain (http://grch37.ensembl.org/Homo_sapiens/Transcript/ProteinSummary?db=core;g=ENSG00000112655;r=6:43100264-43129457;t=ENST00000489707).

Generally, the overall **PTK7** expression was lower in ACC than in normal adrenal glands (Figure 1C). However, the ENST00000489707.5 transcription was similar between the tumor and normal tissues (Figure 1D). By calculating the

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**Figure 1.** **PTK7** transcript profile in ACC and normal adrenal gland. (A) The log2 (TPM) expression of each **PTK7** transcript in ACC and normal adrenal gland. (B) The isoform percentage of each transcript in ACC and normal adrenal gland. (C) Comparison of total **PTK7** expression between ACC and normal adrenal gland. (D) Comparison of ENST00000489707.5 expression between ACC and normal adrenal gland. (E) Comparison of ENST00000489707.5 isoform percentage between ACC and normal adrenal gland. Data in tumor and normal tissues were obtained from TCGA-ACC and GTEx-adrenal gland, respectively.
Figure 2. Comparison of 2 PTK7 isoform percentages in 5 types of cancer. (A) A heatmap showing the percentage of ENST00000489707.5 and ENST00000230419.8 in ACC (N=77) and in other 4 cancers: prostate cancer (PRAD, N=495), lung adenocarcinoma (LUAD, N=513), colon cancer (COAD, N=286), and breast cancer (BRCA, N=1091). (B, C) Plot charts comparing the percentage of ENST00000230419.8 (B) and ENST00000489707.5 (C) in ACC, PRAD, LUAD, COAD, and BRCA. The isoform percentage data was obtained from TCGA. IsoPct – isoform percentage.
To get a more robust estimation, we treated ENST00000489707.5 expression as a continuous variable in univariate and multivariate Cox analysis. Advanced pathological stages, with residual tumors, Weiss venous invasion, invasion of tumor capsule, and ENST00000489707.5 expression were risk factors of shorter PFS (Table 1). ENST00000489707.5 expression showed independent prognostic value after adjustment for the other 4 factors (HR: 1.227, 95%CI: 1.077–1.398, \(p=0.002\)) (Table 1).

When making risk estimation in terms of DSS, advanced pathological stages, with residual tumors, Sinusoid invasion, Weiss venous invasion, invasion of tumor capsule, and ENST00000489707.5 expression were risk factors of shorter DSS (Table 2). ENST00000489707.5 expression was also independently associated with unfavorable DSS after adjustment for the other 5 factors (HR: 1.419, 95%CI: 1.154–1.745, \(p=0.001\)) (Table 2).

**ENST00000489707.5 expression was associated with PTK7 DNA methylation status in ACC**

Using DNA methylation data from Infinium Human Methylation 450K BeadChip based on 77 ACC cases, we examined whether ENST00000489707.5 expression was influenced by the methylation status of CpG sites in PTK7 gene locus. In the array, the methylation status of 26 CpG sites in the gene locus was measured (Figure 4A). Since ENST00000489707.5 is the dominant transcript of PTK7 in ACC, we confirmed a strong positive correlation between ENST00000489707.5 and PTK7 expression (Figure 4A). By setting the median level of correlation (Pearson’s \(r\geq0.4\)) as the cutoff, we found that cg20819617 methylation was negatively correlated with both PTK7 and ENST00000489707.5 expression (Figure 4B). Therefore, we infer that cg20819617 is a critical CpG site modulating PTK7 transcription.
**Discussion**

Although some previous studies explored the functional role and prognostic value of PTK7 expression in some cancers, most of them generally considered total PTK7 expression rather than the expression of different isoforms with different functions. In cancer cells, the full-length PTK7 protein enhances the Akt and c-Jun signaling and weakens the p53 signaling [17]. However, it decreases ERK and CREB-mediated gene expression [17]. sPTK7 activates the RhoA and p38 pathway, thereby modulating the expression of migration-related genes [15,17]. cPTK7 can be transferred into the nucleus, leading to enhanced phosphorylation of ERK1/2 and the phosphorylation of CREB at Ser133 and ATF1 at Ser63. Therefore, cPTK7 activates the RAS/ERK pathway and enhances the transcriptional activity of CREB and ATF1 [17]. cPTK7 can also upregulate the expression of cadherin-11 (CDH11), a pro-migratory gene [17]. Therefore, cPTK7 might have some opposite regulatory effects compared to the full-length PTK7 [17]. However, another recent study showed that cPTK7 has biological activity in activating the canonical Wnt-signaling during early Xenopus development, which is similar to the full-length PTK7 protein, by elevating the expression of LRP6 protein [21]. Therefore, the PTK7 variants might have different activities in different tissues, which contribute to the functional diversity of this protein. We infer that the expression of certain variants rather than total PTK7 expression could be specific biomarkers in typical cancers.

**Table 1. Univariate and multivariate analysis of PFS in patients with ACC.**

| Parameters                        | Univariate analysis |                      | Multivariate analysis |
|-----------------------------------|---------------------|----------------------|-----------------------|
|                                   | p       | HR     | 95% CI (lower/upper) | p       | HR     | 95% CI (lower/upper) |
| Age                               | 0.973   | 1.000  | 0.983   | 1.018  |        |                      |
| Sex                               |         |        |         |        |        |                      |
| Male (N=32)                       |         |        |         |        |        |                      |
| Female (N=60)                     | 0.116   | 1.646  | 0.885   | 3.062  |        |                      |
| Pathological stages               |         |        |         |        |        |                      |
| III/IV (N=37)                     |         |        |         |        |        |                      |
| I/II (N=53)                       | <0.001  | 0.223  | 0.121   | 0.411  | 0.442  | 0.656   | 0.224  | 1.922  |
| Nuclear grade III/IV              |         |        |         |        |        |                      |
| Present (N=56)                    |         |        |         |        |        |                      |
| Absent (N=15)                     | 0.982   | 1.009  | 0.475   | 2.143  |        |                      |
| Residual tumor                    |         |        |         |        |        |                      |
| Yes (N=19)                        |         |        |         |        |        |                      |
| No (N=64)                         | <0.001  | 0.202  | 0.106   | 0.386  | 0.098  | 0.433   | 0.161  | 1.166  |
| Sinusoid invasion                 |         |        |         |        |        |                      |
| Yes (N=29)                        |         |        |         |        |        |                      |
| No (N=39)                         | 0.206   | 0.651  | 0.335   | 1.266  |        |                      |
| Weiss venous invasion             |         |        |         |        |        |                      |
| Yes (N=36)                        |         |        |         |        |        |                      |
| No (N=43)                         | 0.016   | 0.474  | 0.257   | 0.872  | 0.783  | 0.898   | 0.417  | 1.934  |
| Invasion of tumor capsule         |         |        |         |        |        |                      |
| Yes (N=48)                        |         |        |         |        |        |                      |
| No (N=35)                         | 0.014   | 0.445  | 0.234   | 0.847  | 0.237  | 0.626   | 0.289  | 1.359  |
| ENST00000489707.5 expression      | 0.001   | 1.227  | 1.090   | 1.381  | 0.002  | 1.227   | 1.077  | 1.398  |
Table 2. Univariate and multivariate analysis of DSS in patients with ACC.

| Parameters                        | RFS univariate analysis | RFS multivariate analysis |
|-----------------------------------|-------------------------|---------------------------|
|                                   | p          | HR (95% CI lower/upper)  | p          | HR (95% CI lower/upper)  |
| Age                               | 0.553      | 1.007 (0.985/1.029)    |            |                          |
| Sex                               |            |                         |            |                          |
| Male (N=30)                       | 1.000      |                          |            |                          |
| Female (N=60)                     | 0.585      | 1.243 (0.569/2.716)    |            |                          |
| Pathological stages               |            |                         |            |                          |
| III/IV (N=36)                     | 1.000      |                          |            |                          |
| I/II (N=53)                       | <0.001     | 0.121 (0.051/0.287)    | 0.472      | 1.908 (0.328/11.094)     |
| Nuclear grade III/IV              |            |                         |            |                          |
| Present (N=54)                    | 1.000      |                          |            |                          |
| Absent (N=15)                     | 0.951      | 0.971 (0.385/2.450)    |            |                          |
| Residual tumor                    |            |                         |            |                          |
| Yes (N=18)                        | 1.000      |                          |            |                          |
| No (N=64)                         | <0.001     | 0.065 (0.036/0.196)    | 0.002      | 0.062 (0.010/0.368)      |
| Sinusoid invasion                 |            |                         |            |                          |
| Yes (N=28)                        | 1.000      |                          |            |                          |
| No (N=38)                         | 0.039      | 0.412 (0.178/0.957)    | 0.986      | 0.989 (0.299/3.277)      |
| Weiss venous invasion             |            |                         |            |                          |
| Yes (N=35)                        | 1.000      |                          |            |                          |
| No (N=42)                         | 0.005      | 0.325 (0.148/0.713)    | 0.616      | 0.706 (0.182/2.746)      |
| Invasion of tumor capsule         | 1.000      |                          |            |                          |
| Yes (N=47)                        | 0.018      | 0.351 (0.147/0.835)    | 0.089      | 0.396 (0.136/1.151)      |
| No (N=34)                         |            |                         |            |                          |
| ENST00000489707.5 expression      | 0.002      | 1.266 (1.088/1.473)    | 0.001      | 1.419 (1.154/1.745)      |

In this study, we found that an alternative transcript, ENST00000489707.5, which only encodes an extracellular Ig domain and an intracellular kinase domain, is the dominant isoform of PTK7 in both ACC and normal adrenal glands. Its expression percentage was significantly higher in ACC than in normal adrenal glands. In addition, we showed that ACC tissues had preferred expression of this transcript compared with other cancers, suggesting the expression of PTK7 transcripts is tissue-specific. Prognostic analysis showed that ENST00000489707.5 had independent prognostic value in PFS (HR: 1.227, 95%CI: 1.077–1.398, p=0.002) and DSS (HR: 1.419, 95%CI: 1.154–1.745, p=0.001) after adjustment for other risk factors. Therefore, ENST00000489707.5 expression might serve as a specific prognostic marker in ACC patients.

Currently, no study has explored the functional role of the protein encoded by ENST00000489707.5. This protein only has some Ig-like domains and the cytoplasmic tyrosine kinase domain, lacking the transmembrane region and the juxtamembrane region compared to the full-length protein. Therefore, it is unlikely to be proteolyzed by ADAM17 and γ-secretase to generate cPTK7. Available evidence suggests that abnormal activation of the Wnt/β-catenin signaling and p53/Rb inhibition are 2 critical drivers of ACC [22,23], while the CREB protein is decreased during ACC development [24,25]. Therefore, we hypothesized that the protein encoded by ENST00000489707.5 contributes to ACC development via functions similar to those of full-length PTK7, but not cPTK7. However, future molecular studies are required to validate this hypothesis.
Figure 4. Methylation profiles of 26 CpG sites in PTK7 gene locus. (A) A heatmap showing PTK7 and ENST00000489707.5 expression and the methylation profile of 26 CpG sites in PTK7 gene locus. (B) Pearson’s correlation coefficients between the methylation status of each CpG site and PTK7/ENST00000489707.5 expression.
By using DNA methylation data in TCGA-ACC, we also identified a critical CpG site (cg20819617) that showed a moderately negative correlation with ENST00000489707.5 expression and total PTK7 expression. Since ENST00000489707.5 is a splicing product of PTK7, we hypothesized that the hypomethylation status of this CpG site facilitates PTK7 transcription and subsequently increases accumulation of splicing products.

Conclusions

ENST00000489707.5 is a preferred alternative splicing product of PTK7, with a significantly increased proportion in ACC compared with other cancers. Its expression shows potential prognostic value in terms of PFS and DSS in ACC patients. The methylation status of cg20819617 might play a critical role in modulating PTK7 transcription and ENST00000489707.5 expression.

Conflict of interest

None.

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