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ORIGINAL ARTICLE

ENTPD5: identification of splicing variants and their impact on cancer survival

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Abstract

NTPDase5 is a nucleotidase of the endoplasmic reticulum that plays an important role in proteostasis as a regulator of protein N-glycosylation. This enzyme was first identified in hamster as a proto-oncogene activated upon a single nucleotide deletion that causes a frameshift leading to a truncated protein. Truncated NTPDase5 proteins were detected in human samples, but an oncogene was never identified. Searching for transcript variants in the GenBank database and using TCGA data, we discovered that splice variants could originate truncated human NTPDase5 proteins. We identified three main splicing events in the *ENTPD5* gene: alternative acceptors, exon skipping, and alternative terminators. The analysis of impact of splicing events in cancers showed that skipping of exon 11—the event that leads to truncated proteins similar in size to the hamster oncogene—does not affect the hazard ratio of most tumors and was, in fact, a protective factor in the only two cancer studies where it was significant. We also identified four main patterns of impact of *ENTPD5* in cancer and a potential variant-specific regulation by miR-215. Our findings shed light on a two-decade uncertainty about the origin of truncated NTPDase5 and contribute to the characterization of its impacts in cancer.

Keywords *ENTPD5* · NTPDase5 · Splicing events · Transcript variants

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Introduction

The ectonucleoside triphosphate diphosphohydrolase (NTPDase) family of enzymes is composed of eight paralogs in humans involved in the degradation of di- and triphosphate nucleotides [1]. NTPDase5, specifically, is an endoplasmic reticulum enzyme whose catalytic site is directed to the lumen [2, 3]. The main substrate of NTPDase5 is UDP which is converted to UMP with the release of inorganic phosphate, albeit it also hydrolyzes other diphosphate nucleotides to a lesser extent [4]. In addition to the intracellular localization, it has already been shown that NTPDase5

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can be secreted as a soluble enzyme upon cleavage of the signal peptide sequence [5, 6] and therefore could regulate UDP-binding receptors [7]. One of the established roles of NTPDase5 is in the N-glycosylation of proteins in the endoplasmic reticulum. During this process, misfolded proteins need to be glycosylated by UDP-glucose:glycoprotein glucosyltransferase (UGGT) in order to recruit calnexin and calreticulin for another attempt of proper folding. The substrate for UGGT is UDP-glucose, which enters the endoplasmic reticulum through an antiport mechanism with UMP produced by NTPDase5 [3].

NTPDase5 has also been associated with cancer development in several models. It has been identified as a mediator of pro-tumoral effects of AKT—namely stimulation of the Warburg effect [3, 8]—and p53 variants [9]. It is also known that NTPDase5 is involved in the process of white matter invasion of glioblastomas [10], regulation of liver metabolism [11], and resistance to apoptosis [12–14].

In Syrian hamster, a single nucleotide deletion at the codon 214 of the NTPDase5 gene leads to the production of a 246 amino acid (~27 kDa) truncated protein containing the first 213 amino acids of NTPDase5 and 33 amino acids from the altered reading frame produced by the deletion, which is considerably smaller than the 469 amino acids (~47 kDa) of the wild-type NTPDase5. This truncated protein lacks enzymatic activity [4] and was described as the oncogene mt-PCPH [15], which has transforming activity by cooperating with Ras to produce sustained activation of ERK1, a kinase intimately involved in cell proliferation [16]. In humans, peptides of unknown origin equivalent in molecular size to mt-PCPH (~27 kDa) have been detected in both normal tissue and tumor cell lines through Western blotting using anti-serum against the hamster protein [17, 18] which made several authors hypothesize that NTPDase5 could also be a proto-oncogene in humans. It is not clear, however, whether these related proteins play a role in the malignant transformation of human cells, as the literature presents conflicting data about their expression in normal and tumor cells. For example, while benign breast tissue expresses only the 47-kDa peptide, fragments other than the wild-type protein were detected in breast carcinomas [19]. In contrast, another study showed only the presence of 27-kDa peptides, related to mt-PCPH, in extracts from epithelial cells derived from normal breast [18].

Some pro-tumor roles of mt-PCPH have been found in human tumor cell lines with ectopic expression of the hamster oncogene. In most of them, mt-PCPH appears to have similar effects to the wild-type NTPDase5 protein but with greater intensity [13, 20, 21]. However, as mt-PCPH was heterologously expressed in these works and there are no functional studies of the variants of endogenous NTPDase5 found in human cells, the biological significance of peptides related to mt-PCPH in humans is still unknown. Also, some of these studies attributed the pro-tumoral effects of mt-PCPH to the depletion of intracellular ATP levels [12, 21]. This effect was later very well characterized for the canonical full-length NTPDase5 through the identification of the ENTPD5/CMPK1/AK1 cycle which is dependent on its enzymatic activity [3]. However, mt-PCPH does not show enzymatic activity in situ [4]. Since *ENTPD5* is a unique gene in mammals, some authors suggest that these other detected peptides could be splice variants [18, 19, 22] but no evidence was presented so far. Considering the increasing interest in the study of NTPDase5 as a potential target for cancers and the uncertainty about the origin and relevance of its non-canonical peptides, the aim of this article was to analyze transcripts of the human *ENTPD5* gene in order to identify possible correspondents to mt-PCPH and also access the impact of non-canonical transcripts in the overall survival of cancer patients. Searching for transcript variants in the GenBank database and using TCGA data, we discovered that splice variants could originate truncated human NTPDase5 proteins. We describe here the splicing events that occur in transcripts of the *ENTPD5* gene, four main patterns of impact of *ENTPD5* in cancer, and a potential variant-specific regulation by miR-215.

Results

Identification of splicing variants

Considering the uncertainty about the origin of non-canonical NTPDase5 peptides in humans, we decided to investigate potential sequence variations or splicing variants of *ENTPD5*. First, we queried DNA sequencing data from the TCGA PanCancer Atlas database using cBioPortal and found that truncating variants in the *ENTPD5* gene are infrequent. Out of 10,953 patients from 32 different studies, only 64 variations were detected in 19 types of cancer. Variations were encountered throughout the sequence of the *ENTPD5* gene with no particular hotspot site. The cancer study with the highest amount of variants was uterine corpus endometrial carcinoma (15 patients) followed by skin cutaneous melanoma with 12 samples. Variants in other studies were encountered in five or less patients. Considering all studies, the most common type of events are missense variations (43 patients), followed by 15 truncating variations which correspond to less than 0.2% of patients. The other six variations involve gene fusion. Since non-canonical peptides were frequently described in earlier studies [17–19, 23], it is highly unlikely that the majority of those events could be explained by truncating variations taking into account their very low prevalence.

Then, we looked for transcript variants of the human *ENTPD5* in order to investigate whether splicing variants

could be identified. Interestingly, the NCBI Protein database has 31 entries predicted from transcript sequences, 17 of them equivalent to the canonical protein, which has 428 amino acids, while the others included seven unique proteins ranging from 236 to 410 amino acids. Aligning the consensus human *ENTPD5* gene to the mRNA sequences corresponding to the eight unique protein entries, we detected alternative use of exons that indicate that these are indeed splicing variants. Using the TCGA SpliceSeq database, we encountered several of those splicing events in samples from human cancers and identified a few others that were not present in the transcripts from the NCBI database. The Ensembl database contains two transcripts that match the sequences deposited in the NCBI Protein database and another transcript that was predicted from TCGA SpliceSeq data. Finally, we looked for evidence of protein detection in the Proteomics DB platform and were able to identify three isoforms based on peptides detected using mass spectrometry. Table 1 summarizes the identified variants and correspondent splicing events.

The human *ENTPD5* gene has 23 exons. Previous comparisons of the sequence of NTPDase5 with its orthologs and paralogs revealed important conserved domains and residues. NTPDase5 has all the five apyrase-conserved regions (ACR) found from yeast to superior eukaryotes and two out of the four conserved regions (CR) among mammalian NTPDases. In addition, three putative N-glycosylation sites and four cysteine residues predicted to form disulfide bond were described [24] (Fig. 1).

We identified that the coding sequence of *ENTPD5* starts at the exon 5, may use different acceptors for exon 6 (referred to as exons 6.1, 6.2, and 6.3), and uses one exon among exons 17 to 21 as an alternative terminator. In addition, skipping of exons 11, 13, and/or 14 may also happen in some transcripts. Additionally, skipping of exons 2 and 4 in the 5′ UTR of *ENTPD5* were also detected (Table 2) but were not included in the following analyses since they do not alter the protein sequence.

Both alternative acceptors to exon 6 result in putative truncated proteins that share the same amino acid sequence of the canonical NTPDase5 for 73 residues and 18 or 5 unconserved residues when exon 6.1 or 6.3 are used, respectively. These truncated proteins would miss several important domains and residues such as four out of the five apyrase conserved regions (ACR2-5), the two conserved regions shared among mammalian NTPDases (CR2 and 4), two asparagine residues predicted to be sites of N-glycosylation, and all four cysteine residues involved in putative disulfide bonds. The absence of these features probably abrogates the enzyme activity.

Alternative use of terminators appears to also be an important feature of *ENTPD5*. The transcripts deposited in the NCBI database indicate the use of five different terminators (exons 17 to 21). These variants share the same first 400 amino acids and only differ in their final 2 to 28 residues. Since most residues are conserved, these variants retain all the important domains and residues identified in the literature so far with the exception of the ACR5 domain which is only found in the sequence of exon 17. Therefore, transcripts that use exons 18 to 21 only have the first four ACR which could affect the enzymatic activity of these variants.

Table 1 Putative variants of NTPDase5 based on protein isoforms and transcripts detected and predicted

Level of evidence	Accession	Protein length	Splicing event(s)[a]	Impact in the protein sequence [b]
Protein detected	Proteomics DB O75356	428 aa	AA6.2, AT17	None (canonical protein)
	Proteomics DB G3V4I0	407 aa	AA6.2, AT19	Missing ACR5
	Proteomics DB G3V3Y0	91 aa	AA6.1	Truncated protein – missing CR2-4, ACR2-5, conserved Asn and Cys residues
Transcript detected	NCBI Protein NP_001369187.1	410 aa	AA6.2, AT21	Missing ACR5
	NCBI Protein NP_001308913.1	405 aa	AA6.2, AT18	Missing ACR5
	NCBI Protein NP_001369191.1	402 aa	AA6.2, AT20	Missing ACR5
	NCBI Protein XP_016877305.1	381 aa	AA6.2, ES14, AT17	Missing conserved Cys residue
	NCBI Protein XP_016877306.1	360 aa	AA6.2, ES14, AT19	Missing conserved Cys residue and ACR5
	NCBI Protein EAW81150.1	236 aa	AA6.2, ES11	Truncated protein
Transcript predicted	TCGA SpliceSeq *ENTPD5*	394 aa	AA6.2, ES13, AT17	Missing conserved Cys residue
	TCGA SpliceSeq *ENTPD5*	373 aa	AA6.2, ES13, AT19	Missing conserved Cys residue and ACR5
	TCGA SpliceSeq *ENTPD5*	239 aa	AA6.2, ES11, ES13	Truncated protein – missing CR4, ACR5, conserved Asn and Cys residues
	TCGA SpliceSeq *ENTPD5*	78 aa	AA6.3	Truncated protein – missing CR2-4, ACR2-5, conserved Asn and Cys residues

[a]*AA*, alternative acceptors; *AT*, alternative terminators; *ES*, exon skipping

[b]*ACR*, apyrase conserved region; *CR*, conserved region

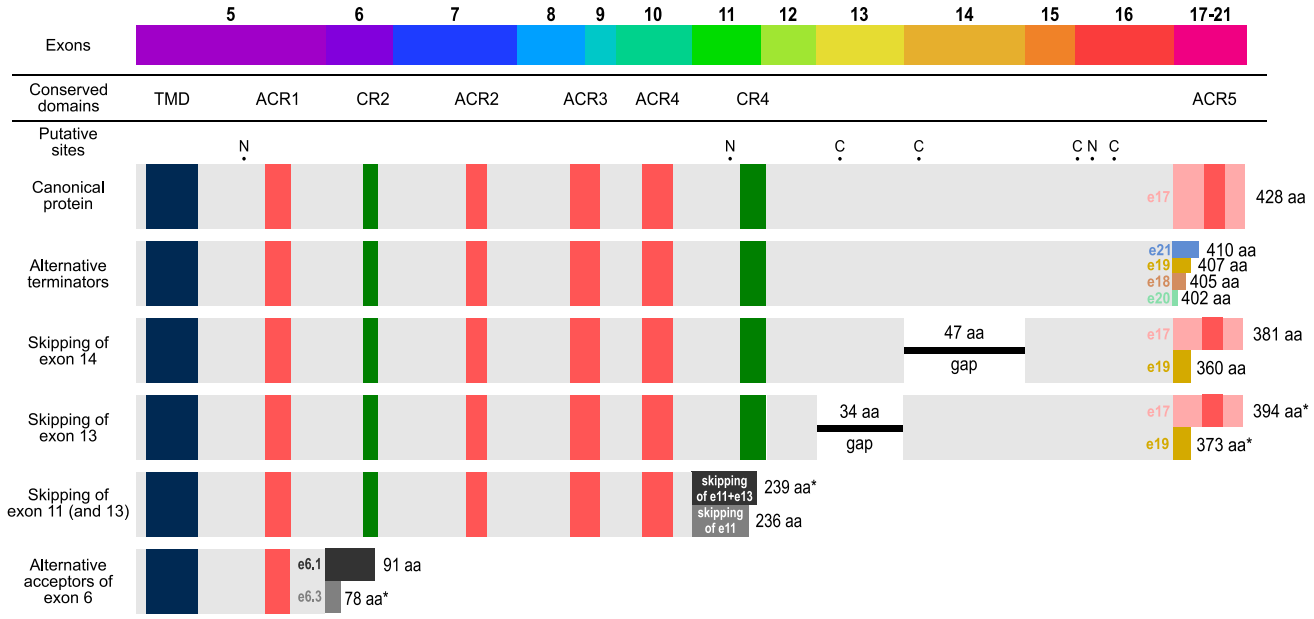


Fig. 1 Schematic representation of the exons of the *ENTPD5* gene aligned to the predicted protein variants. The coding sequence of *ENTPD5* starts at the exon 5 (the UTRs are not shown). Conserved domains and putative sites of N-glycosylation (N) and conserved cysteines (C) involved in disulfide bonds are shown. For the proteins, light grey regions represent conserved residues; dark blue indicates the transmembrane domain (TMD); dark red indicates apyrase conserved regions (ACR); dark green represents conserved regions (CR) of NTPDases; dark shades of grey indicate unconserved residues of the truncated proteins; light colors represent different alternative terminators. *=transcript predicted

Skipping of exon 13 remains in-frame and results in a deletion of 34 residues which includes one conserved cysteine residue expected to be involved in disulfide bonds. Similarly, skipping of exon 14 also maintains the reading frame and causes a 47 amino acid gap that misses a conserved cysteine [24]. Skipping of exon 11 or both 11 and 13, however, results in a frameshift and a premature stop codon usage. Transcripts that skip exon 11 are predicted to encode a 236 amino acid truncated protein while skipping both exons 11 and 13 encodes a 239 amino acid protein. It is interesting to note that these variants are similar in length to the hamster mt-PCPH that has 246 amino acids. Strikingly, both hamster and human truncated proteins differ from their full-length counterparts at the exact same amino acid position. While the point deletion in the nucleotide sequence of hamster NTPDase5 causes a frameshift and an amino acid change from serine to arginine at residue 214 [15], in humans, this position corresponds to the first amino acid after exon 10, which is a glutamic acid encoded by either exon 11 or 12. Surely, the subsequent residues of human and hamster truncated proteins are not conserved since the human transcripts contain the sequence of exon 12 while the hamster transcript only skips a nucleotide from the hamster-equivalent to the human exon 11.

Frequency of splicing events in human cancers

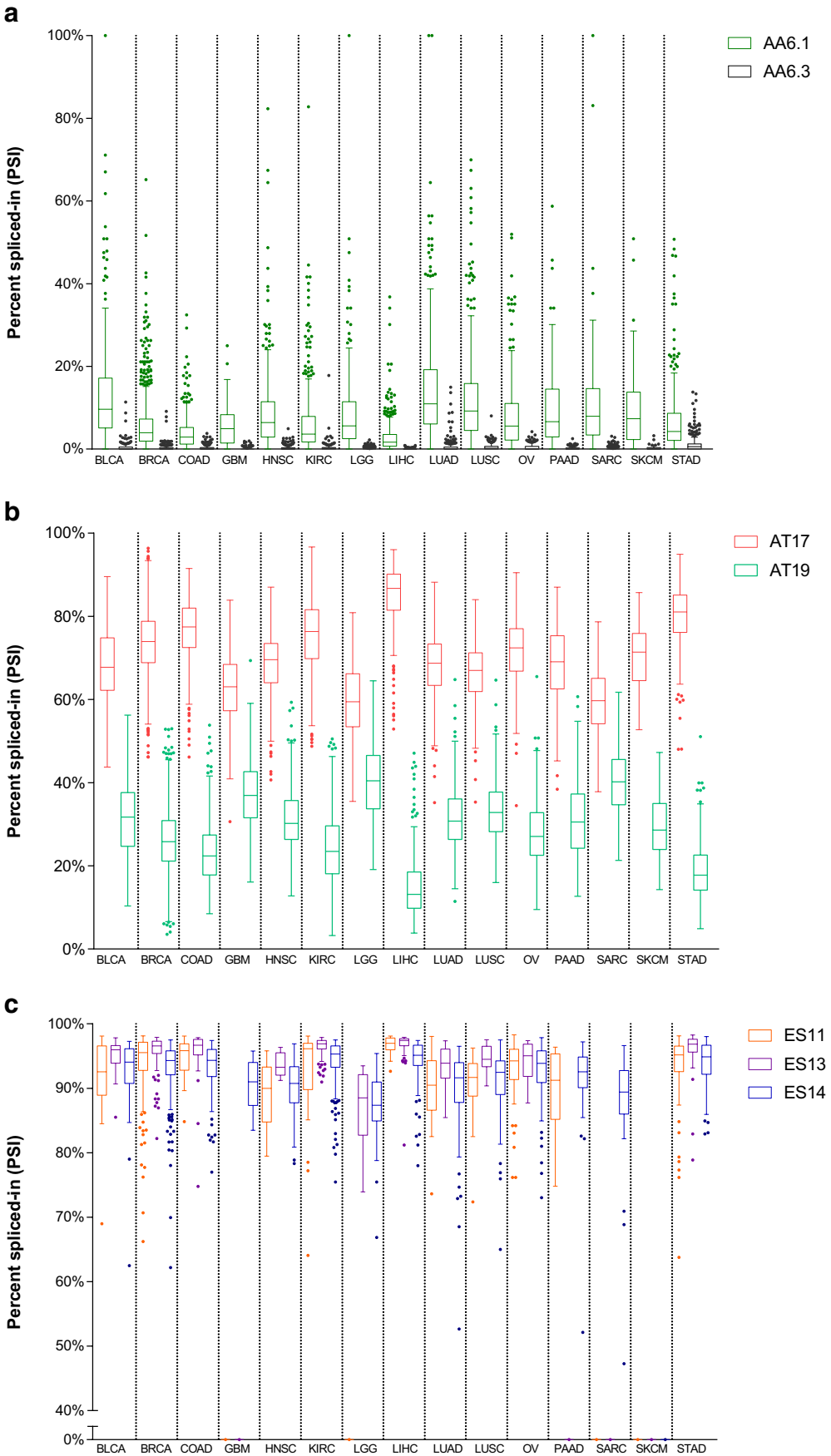
Using the TCGA SpliceSeq database, we identified that splicing events of *ENTPD5* transcripts appear to occur in different frequencies in human cancers depending on the tissue of origin and type of tumor (Table 2). Alternative use of exon 6.1 could be detected in samples from 85.1% of patients and in all the 15 types of cancer analyzed (Table 2). The median percentage of reads with this splicing event (PSI, percent-splice in), however, ranged from 3.6 to 10.9% in kidney renal clear cell carcinoma (KIRC) and lung adenocarcinoma (LUAD), respectively (Fig. 2A). Nonetheless, many outliers were detected and a few patients even had PSI values of 100%. On the other hand, although alternative use of exon 6.3 was detected in all samples (Table 2), the median PSI values were lower than 0.6% (Fig. 2A).

Five alternative terminators for *ENTPD5* were encountered in transcripts deposited in the NCBI Protein database; however, only the use of exons 17 and 19 could be detected in the TCGA SpliceSeq database, where they were present in all samples (Table 2). Together, these two terminators account for more than 99% of all reads (Fig. 2B). Therefore, the other terminators probably could not be detectable above the noise threshold. Accordingly, only protein isoforms containing the amino acid sequences coded by exons 17 and 19 were deposited so far in the Proteomics DB (Table 1). Exon 17 is the preferred terminator for all the investigated studies, reaching a median of 86.7% in liver hepatocellular carcinoma (LIHC). However, in some tumors, the difference is not so substantial, such as in lower-grade glioma (LGG), where a median of 40.4% of reads used exon 19 instead.

Table 2 Frequency of samples with each splicing event among cancer studies

Study	*n*	Samples with AA6.1	Samples with AA6.3	Samples with AT17	Samples with AT19	Samples with ES11	Samples with ES13	Samples with ES14	Samples with ES2	Samples with ES4
BLCA	406	281 (69.2%)	406 (100%)	406 (100%)	406 (100%)	18 (4.4%)	19 (4.7%)	41 (10.1%)	ND	225 (55.4%)
BRCA	1094	986 (90.1%)	1094 (100%)	1094 (100%)	1094 (100%)	152 (13.9%)	165 (15.1%)	385 (35.2%)	60 (5.5%)	932 (85.2%)
COAD	456	379 (83.1%)	456 (100%)	456 (100%)	456 (100%)	24 (5.3%)	26 (5.7%)	133 (29.2%)	ND	258 (56.6%)
GBM	155	108 (69.7%)	155 (100%)	155 (100%)	155 (100%)	ND	ND	15 (9.7%)	ND	87 (56.1%)
HNSC	501	422 (84.2%)	501 (100%)	501 (100%)	501 (100%)	16 (3.2%)	12 (2.4%)	78 (15.6%)	12 (2.4%)	370 (73.9%)
KIRC	533	496 (93.1%)	533 (100%)	533 (100%)	533 (100%)	30 (5.6%)	120 (22.5%)	442 (82.9%)	22 (4.1%)	442 (82.9%)
LGG	515	362 (70.3%)	515 (100%)	515 (100%)	515 (100%)	ND	10 (1.9%)	59 (11.5%)	ND	345 (67%)
LIHC	371	333 (89.8%)	371 (100%)	371 (100%)	371 (100%)	17 (4.6%)	78 (21%)	149 (40.2%)	ND	253 (68.2%)
LUAD	514	470 (91.4%)	514 (100%)	514 (100%)	514 (100%)	44 (8.6%)	25 (4.9%)	108 (21%)	ND	417 (81.1%)
LUSC	501	467 (93.2%)	501 (100%)	501 (100%)	501 (100%)	34 (6.8%)	23 (4.6%)	120 (24%)	11 (2.2%)	442 (88.2%)
OV	412	377 (91.5%)	412 (100%)	412 (100%)	412 (100%)	39 (9.5%)	25 (6.1%)	146 (35.4%)	44 (10.7%)	334 (81.1%)
PAAD	178	136 (76.4%)	178 (100%)	178 (100%)	178 (100%)	12 (6.7%)	ND	37 (20.8%)	ND	111 (62.4%)
SARC	259	160 (61.8%)	259 (100%)	259 (100%)	259 (100%)	ND	ND	27 (10.4%)	ND	125 (48.3%)
SKCM	103	78 (75.7%)	103 (100%)	103 (100%)	103 (100%)	ND	ND	ND	ND	76 (73.8%)
STAD	412	398 (96.6%)	412 (100%)	412 (100%)	412 (100%)	72 (17.5%)	56 (13.6%)	159 (38.6%)	63 (15.3%)	367 (89.1%)
Total	6410	5453 (85.1%)	6410 (100%)	6410 (100%)	6410 (100%)	458 (7.1%)	559 (8.7%)	1899 (29.6%)	212 (3.3%)	4784 (74.6%)

ND not detected


a
Percent spliced-in (PSI)
100%
80%
60%
40%
20%
0%
AA6.1
AA6.3
BLCA BRCA COAD GBM HNSC KIRC LGG LIHC LUAD LUSC OV PAAD SARC SKCM STAD
b
Percent spliced-in (PSI)
100%
80%
60%
40%
20%
0%
AT17
AT19
BLCA BRCA COAD GBM HNSC KIRC LGG LIHC LUAD LUSC OV PAAD SARC SKCM STAD
c
Percent spliced-in (PSI)
100%
90%
80%
70%
60%
50%
40%
0%
ES11
ES13
ES14
BLCA BRCA COAD GBM HNSC KIRC LGG LIHC LUAD LUSC OV PAAD SARC SKCM STAD

◂**Fig. 2** Percentage of reads with each class of splicing events. **a** Alternative acceptors (AA) of exon 6. **b** Alternative terminators (AT). **c** Exon skipping (ES). BLCA bladder urothelial carcinoma. BRCA breast invasive carcinoma. COAD colon adenocarcinoma. GBM glioblastoma. HNSC head and neck squamous cell carcinoma. KIRC kidney renal clear cell carcinoma. LGG lower grade glioma. LIHC liver hepatocellular carcinoma. LUAD lung adenocarcinoma. LUSC lung squamous cell carcinoma. OV ovarian serous cystadenocarcinoma. PAAD pancreatic adenocarcinoma. SARC sarcoma. SKCM skin cutaneous melanoma. STAD stomach adenocarcinoma

Additionally, we identified three exon skipping events that affect the coding sequence of *ENTPD5*. Skipping of exons 11, 13, and 14 were detected only in a fraction of patients—7.1%, 8.7%, and 29.6%, respectively—and not in all tumor types (Table 2). Interestingly, however, the PSI for the samples where those events are detected is usually high (Fig. 2C). Variations in the splicing sites of human *ENTPD5* were detected in only four patients of the TCGA PanCancer Atlas database, which corresponds to less than 0.04% of patients and indicates that the alternative use of exons appears to be mainly a result of differential mRNA processing and not variations in acceptor or donor sites.

Prediction of the impact and regulation of alternative terminators

A transcript variant of the paralog enzyme NTPDase3 that uses an alternative terminator that lacks ACR5 and does not have enzymatic activity was already described. Interestingly, this variant named NTPDase3β negatively interferes with the protein levels of the canonical and functional NTPDase3α variant [25]. Since we encountered that the ACR5 sequence of *ENTPD5* is only found in exon 17 which is subject to alternative splicing, we were interested to know if the use of exon 19 as an alternative terminator impacted the global levels of *ENTPD5* in a similar way to the *ENTPD3* variant. For this, we first correlated *ENTPD5* mRNA levels with the PSI of exons 17 and 19. We found a significant association in 12 out of the 15 cancers analyzed. In all of them, use of exon 17 was positively correlated ($0.16 < r < 0.68$) while there was a negative correlation with exon 19 ($-0.68 < r < -0.17$) (Fig. 3A). The absolute correlation values were similar between variants for all cancers since alternative terminators are mutually exclusive and together exons 17 and 19 account for more than 99% of reads. The correlation of *ENTPD5* levels with the PSI of exons 17 and 19 when all tumors are considered is 0.61 and −0.62, respectively (Fig. 3B–C). This suggests a strong relationship between the absolute levels of *ENTPD5* and the type of alternative terminator used.

In the search of functional impacts of alternative splicing events and also considering the existence of five different terminators and, hence, five different 3′UTR sequences in *ENTPD5* transcripts, we decided to evaluate the potential of differential miRNA regulation of transcript variants. In order to investigate this, we used the miRDB target prediction database to identify potential binding sites for miRNAs and then looked for the correlation levels of the identified miRNAs with *ENTPD5* levels. We decided to also include exons of the 5′UTR and coding sequence (CDS) of *ENTPD5* as there is growing evidence that they can also be targeted by miRNAs [26]. Using the suggested score of 80 as a cut-off for likely target sites, we encountered 23 potential miRNAs that could bind to exons involved in alternative splicing (Supporting Information 2). One of the target sites is in the 5′UTR, four of them are in the CDS, and the remaining 18 are encountered in the 3′UTR of three different terminators, exons 17, 19, and 21. Using miRCancerdb, we found expression data for only ten of these miRNAs in the TCGA database. Nine miRNA regulators had weak correlations ($-0.25 < r < 0.23$) with *ENTPD5* mRNA levels and the sign of the correlation coefficient was not consistent among tumor types. However, miR-215, which was found as a potential target in exon 19, presented positive correlations in all significant tumors and had a stronger range of correlation coefficients in comparison with the other potential miRNAs ($0.22 < r < 0.41$). Since this miRNA potentially targets exon 19, we evaluated the correlation of miR-215 and the PSI of exon 19 in all tumors. Interestingly, there is a negative correlation of −0.44, which corroborates with the target prediction and suggests that miR-215 is a potential negative regulator of *ENTPD5* transcripts that use exon 19 as an alternative terminator (Fig. 3D). Since the use of exon 19 itself is negatively correlated with *ENTPD5* levels, this probably explains why miR-215 is positively correlated with global *ENTPD5* mRNA. It is interesting to note that the distribution of data in the miR-215 vs. PSI AT19 correlation resulted in two big populations: most samples had lower levels of miR-215 and higher levels of PSI for exon 19, while a smaller subset of samples presented the opposite pattern. Interestingly, this pattern appears to be tumor-specific as the smaller subset of higher miR-215 expression and lower exon 19 usage samples is mainly composed of tumors from the COAD, LIHC, and STAD cohorts.

Impact of splicing events in the overall survival of cancer patients

After the identification of splicing events of *ENTPD5* in human cancers, we were interested in evaluating whether these events were related to the malignancy of tumors since NTPDase5 peptides appeared to be differentially expressed in normal and tumor samples. For this, we selected the 15 solid tumors included in the TCGA database with the highest number of deceased patients. Studies with only non-significant results are presented in Supporting Information 3. First, we assessed the impact of global levels of *ENTPD5* on survival

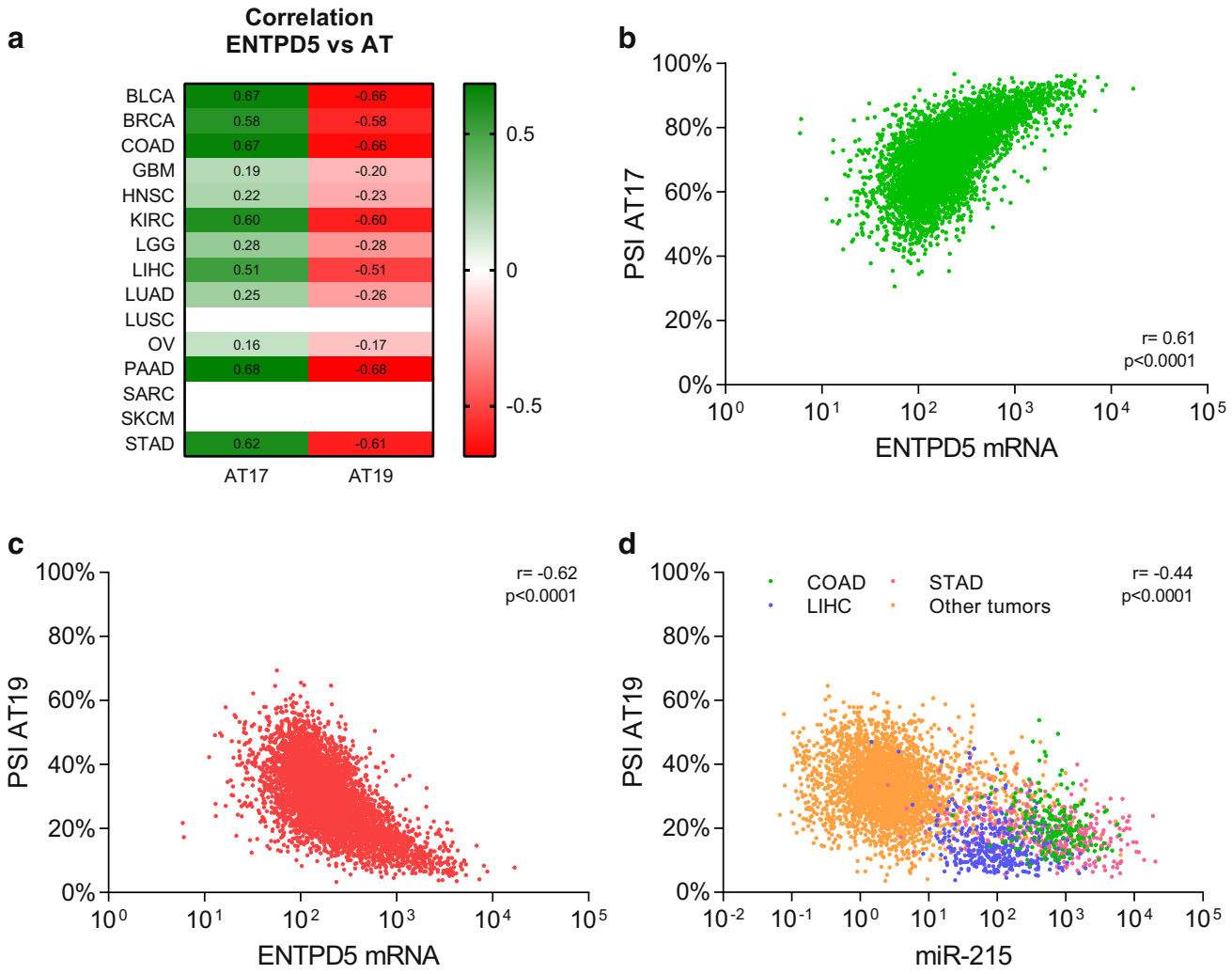


Fig. 3 Correlation of alternative terminators and *ENTPD5* and *miR-215*. **a.** Heat map showing the correlation of *ENTPD5* mRNA levels with the PSI of alternative terminators 17 and 19 for 15 cancers. Blank cells indicate non-significant values. **b–c** Correlation of ENTPD5 mRNA levels with the PSI of exons 17 and 19 for all tumors samples combined. **d** Correlation of miR-215 with the PSI of exon 19 for all tumors samples combined. Spearman's correlation coefficients are shown

using the Cox proportional-hazard model and identified that after adjusting for clinical variables, a one-unit increase on the z-score expression of *ENTPD5* increased the risk of death by 17% in LGG (HR 1.17, IC 95% 1.06–1.43) and 23% in SARC (HR 1.23, IC 95% 1.06–1.43). However, for the tumors COAD and KIRC, the effect was on the opposite direction, with a 46% and 31% reduction on the risk of death (HR 0.54, IC 95% 0.33–0.88, and HR 0.69 IC 95% 0.55–0.86, respectively) (Fig. 4A). However, when the HR of these tumors is adjusted for the PSI of alternative terminators, global levels of *ENTPD5* only affect the risk of death of tumors in which clinically adjusted *ENTPD5* was a risk factor (HR 1.28, IC 95% 1.05–1.56, and HR 1.23, IC 95% 1.06–1.43, for LGG and SARC, respectively), suggesting that for COAD and KIRC, it is not the expression level of *ENTPD5* per se that is a risk factor, but the higher occurrence of a splicing event.

Next, we looked for the impact of the splicing events of *ENTPD5* in the HR adjusting or not for the impact of global levels of *ENTPD5*. Alternative use of terminators impacted the HR of patients of five studies (Fig. 4B). Similarly to *ENTPD5* global levels, alternative terminators do not affect tumors in the same way. For COAD and KIRC patients, alternative use of exon 17 is a protective factor (HR 0.69, IC 95% 0.54–0.88, and HR 0.68, IC 95% 0.59–0.79, respectively) and use of exon 19 is a risk factor (HR 1.5, IC 95% 1.1–1.9, and HR 1.5, IC 95% 1.3–1.7, respectively). Adjusting the HR of these tumors for the levels of *ENTPD5* does not alter the pattern of impact in the HR. This indicates that the effect of alternative terminators is independent of the ENTPD5 levels in these tumors. Furthermore, alternative use of exon 17 is a risk factor for LGG patients—and use of exon 19, a protective factor—only when not adjusted for

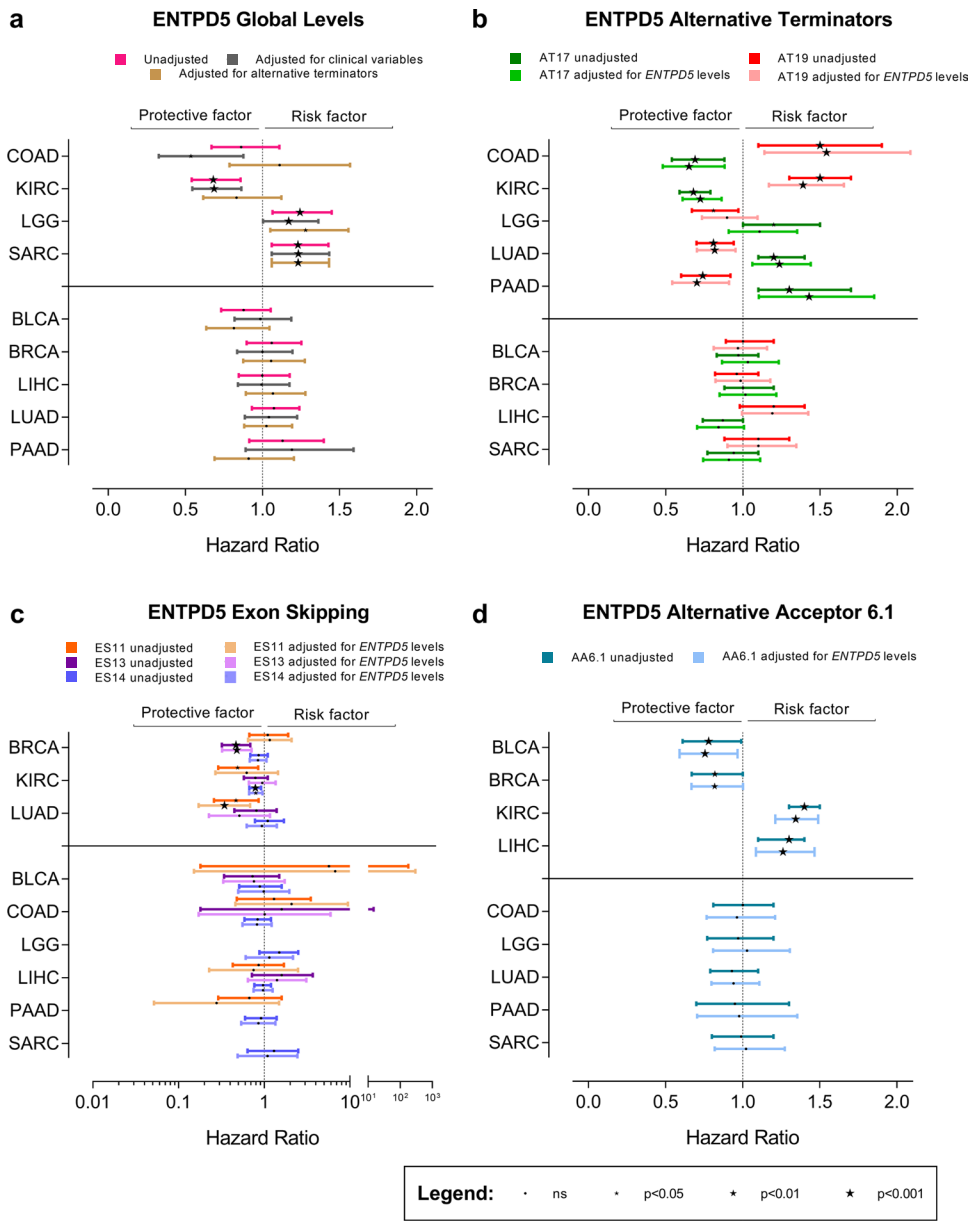


Fig. 4 Cox proportional hazard analysis of overall survival data. The hazard ratio of global mRNA levels of *ENTPD5* is shown in **a**. **b**–**d** indicates the hazard ratios of splicing events. The horizontal line separates significant and not significant results

ENTPD5 levels. Together with SARC, this is indicative that, for these tumors, *ENTPD5* levels are an independent risk factor. Contrastingly, for lung adenocarcinoma (LUAD) and pancreatic adenocarcinoma (PAAD), the use of exon 19 is protective, while use of exon 17 is a risk factor, although global levels of *ENTPD5* did not significantly affect the HR in these studies (Fig. 4B).

We also investigated the hazard ratio of a one-unit increase in the z-score of the PSI of exon skipping events. After adjusting for *ENTPD5* levels, skipping of exon 11 was a protective factor for LUAD patients (HR 0.34; IC 95% 0.17–0.69) (Fig. 4C). This event is related to the encoding of truncated NTPDase5 proteins and was only detected in 8.6% of patients with this tumor (Table 2). In addition, skipping of exon 13 and 14 were protective factors for breast invasive carcinoma (BRCA) (HR 0.48; IC 95% 0.32–0.71) and KIRC patients (HR 0.80; IC 95% 0.67–0.96), respectively (Fig. 4C). These in-frame splicing events were found in 15.1% and 82.9% of samples of the respective studies (Table 2).

The impact of alternative use of exon 6 acceptors was also assessed. Alternative use of exon 6.1 or 6.3 result in putative 91 or 78 amino acid truncated proteins, respectively, that lack most conserved domains and residues. After adjusting for *ENTPD5* levels, increases in the PSI of exon 6.1 were a protective factor for bladder carcinoma (BLCA) and breast invasive carcinoma (BRCA) patients (HR 0.76, IC 95% 0.59–0.97, and HR 0.82, IC 95% 0.67–1.00, respectively), and a risk factor in the KIRC and liver hepatocellular carcinoma (LIHC) studies (HR 1.34, IC 95% 1.21–1.49, and HR

1.26, IC 95% 1.09–1.47, respectively) (Fig. 4D). Increases in the PSI of exon 6.3 in any tumor, on the other hand, did not present a significant HR (Supporting Information 3).

Discussion

Combining the identified transcripts from the NCBI database and the predicted transcripts from the splicing events detected in the TCGA SpliceSeq database together with protein isoform data from Proteomics DB, we provide here the first description of alternative splicing of *ENTPD5*. Of note, in this study, we were able to identify transcripts that encode putative proteins that are remarkably similar in size to the hamster mt-PCPH oncoprotein and the 27-kDa NTPDase5 peptides detected through Western blotting in human samples in previous studies using anti-serum raised in rabbits against a recombinant hamster NTPDase5 [17–19, 23]. Considering that mt-PCPH is catalytically inactive [4] and that these predicted truncated human variants similarly lack the four conserved cysteine residues, CR4 [24], and the putative substrate-binding pocket—predicted from the rat NTPDase2 crystal structure [4, 27]—these variants probably are also inactive. It was previously suggested that mt-PCPH could act through protein–protein interactions instead, since it was predicted that it lacks one of the lobes that shape the catalytic cleft of NTPDases and potentially exposes residues otherwise protected [4]. Further studies are needed to evaluate whether these putative proteins are indeed expressed and their potential functions, if any. It is interesting to note, however, that contrary to hamster, these putative truncated proteins are possibly non-oncogenic in humans since skipping of exon 11 was a protective factor in the tumors where it affected the risk of death and the 27-kDa NTPDase5 protein was detected in several normal tissues [17, 18, 23]. Two other smaller putative truncated proteins resulting from alternative use of acceptors for exon 6 are expected from transcript data and would lack four ACR alongside the other same missing features of mt-PCPH.

In addition, we identified that *ENTPD5* has five different terminators, two of which account for more than 99% of reads in human cancers and were already detected at the protein level. The two most common terminators differ in that the most abundant, exon 17, contains the sequence for the ACR5, a conserved functional site encountered in enzymes with ectonucleotidase activity while the other, exon 19, does not. Interestingly, an increase in the PSI of those terminators affects the risk of mortality in opposite manners. Since this is the first time that these variants are described, it is difficult to address why they affect tumors differently but we hypothesize that the lack of ACR5 in exon 19 may be one of the factors. Although the impact of missing ACR5 is unknown, it was also identified in a NTPDase3 splicing variant—named NTPDase3β—that uses an alternative terminator. This variant is not enzymatically active and also significantly reduces the amount of canonical NTPDase3α that is adequately processed and trafficked to the cell membrane [25]. In our analysis, use of the ACR5-lacking exon 19 was negatively correlated with global levels of *ENTPD5*, which may indicate a regulatory role similar to NTPDase3β of this *ENTPD5* variant. We also encountered a possible site for miR-215 targeting in the sequence of exon 19 that is absent in exon 17 and in the remaining coding sequence of *ENTPD5*. This miRNA correlated negatively with the PSI of exon 19 which suggests that the predicted targeting indeed occurs and is important for the regulation of *ENTPD5* levels since it correlated positively with global levels of mRNA. Interestingly, miR-215-3p has been found dysregulated in many cancers and directly targets the transcript factor FoxM, which is inhibited by FoxO, an inhibitor of *ENTPD5* transcription [3, 28]. A cross-talk between FoxM and FoxO may selectively affect the expression of *ENTPD5* variants. Another potential difference between alternative terminators 17 and 19 is that a complementary sequence to exon 19, but not 17, is partially transcribed in transcripts of the *COQ6* gene in the opposite strand, which could form double-strand RNA structures and generate small RNAs.

Skipping of exons 13 and 14 was also identified in our data. As previously shown, transcripts with these exons spliced out remain in-frame and do not affect conserved functional domains, although they lack conserved cysteines predicted to form disulfide bonds. The effect of these events on the enzymatic activity of NTPDase5 is unknown, although it can be expected that some alteration in the tertiary structure of the proteins should happen due to the residue gap and the missing conserved cysteine. Skipping of exon 14 occurred on 29.6% of all patients, but was especially frequent in kidney renal clear cell carcinoma where it was detected in 82.9% of samples with a median PSI of 95.3% of all reads. This implicates that the 428 amino acid protein, considered as the canonical NTPDase5 in the literature, is not the most abundant variant in this tumor.

Although deregulations in the expression of NTPDase5 have been found in many cancers, their impacts are diverse and often antagonistic. In glioblastoma and testis, prostate, and breast tumors, for example, NTPDase5 levels are only detectable or more highly encountered in malignant tissues in comparison to normal samples [19, 21, 29, 30]. Accordingly, NTPDase5 levels appear to gradually increase during the malignant transformation of breast and prostate cancers [19, 21] and expression of NTPDase5 was associated with shorter survival rates of lung cancer patients [14]. In contrast, however, the opposite pattern is observed in colon and larynx carcinomas where *ENTPD5* mRNA levels and NTPDase5 protein levels, respectively, appear to be progressively lost during carcinogenesis [23, 31]. In

our study, global levels of *ENTPD5* adjusted for clinical variables affected the risk of death of four tumors as either a protective—COAD and KIRC—or a risk factor—LGG and SARC. Corroborating with the literature, *ENTPD5* expression in colon adenocarcinoma also was a protective factor, but surprisingly, the alternative use of exon 17—which has a PSI of 77.4% of reads—appears to be the main responsible for these findings since *ENTPD5* levels do not affect the HR significantly when adjusted for alternative terminators. We also could identify that lower-grade gliomas follow the same pattern of glioblastomas—which are grade IV gliomas—in that *ENTPD5* expression is a risk factor. *ENTPD5* also negatively impacted the HR of sarcoma patients, while it was a protective factor for kidney renal clear cell carcinoma. Similarly to the dual role of global levels of *ENTPD5*, alternative use of acceptors and terminators appears to affect tumors differently, and no pattern of risk or protective factor could be determined when different tumor types are considered. Exon skipping, however, appeared to either be protective or not affect the HR at all. The lower frequency of exon skipping in comparison to other events probably also imposes statistical limitations to better assess their impacts. Nonetheless, we were able to summarize our findings in four main groups of cancer studies: those where the alternative use of exon 17—the most abundant—was a protective factor, those where this feature was a risk factor, those where *ENTPD5* global levels were a risk factor, and those where these characteristics did not affect the risk of death but other splicing events did (Fig. 5).

Our results indicate that there are three main splicing events in the *ENTPD5* gene, namely alternative acceptors for exon 6, exon skipping, and alternative terminators. In addition, we evaluated the impact of global levels of *ENTPD5* and splicing events in the overall survival of cancer patients using data from the TCGA database and found that, similarly to previous studies in the literature, *ENTPD5* and its splicing variants can act as both risk and protective factors depending on the type of tumor. We also described a potential tumor-specific mechanism of transcript variant regulation by miR-215. Together, our findings shed light on a two-decade uncertainty about the origin of non-canonical NTPDase5 proteins and contribute to the literature in the characterization of the dual role of NTPDase5.

The exploratory description of splicing variants of *ENTPD5* and their impact on cancer is limited to publicly available transciptomic data. Protein data is missing for most splicing events, although evidence of the existence of protein isoforms expressing amino acid sequences coded by exons 17 and exon 19 is available—which supports the relevance of our key findings in regard to the potential miR-215 regulation and the impact of terminator usage in the survival of cancer patients. The existence of the remaining transcript variants at the protein level and the subcellular localization and activity of the NTPDase5 isoforms needs to be experimentally confirmed. It would be interesting, for example, to evaluate the role of NTPDase5 isoforms in protein folding and the regulation of intracellular ATP levels, both functions which are dependent on the enzymatic activity and

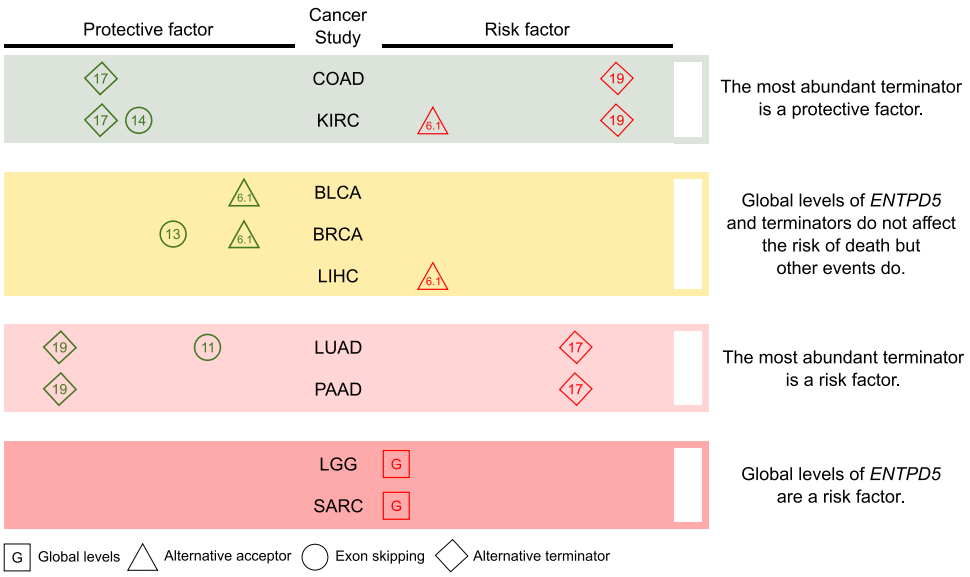


Fig. 5 Summary of Cox proportional-hazard results. Global levels of *ENTPD5* and splicing events are only shown when significant. Cancer studies were grouped in accordance with the patterns of impact of global levels of *ENTPD5* and alternative use of exon 17, the most abundant terminator, on the risk of mortality. COAD colon adenocarcinoma. KIRC kidney renal clear cell carcinoma. HNSC head and neck squamous cell carcinoma. BLCA bladder urothelial carcinoma. BRCA breast invasive carcinoma. LIHC liver hepatocellular carcinoma. LUAD lung adenocarcinoma. PAAD pancreatic adenocarcinoma. LGG lower grade glioma. SARC sarcoma

have been shown to reduce the sensitivity of tumors to endoplasmic reticulum stress conditions and nutrient deprivation [3, 32].

The therapeutic potential of targeting *ENTPD5* was already shown through shRNA studies in breast and pancreatic cancer xenograft models [3, 9]. In addition, small molecule inhibitors of NTPDase5 activity were identified and showed promising anti-proliferative effects against prostate cancer cells [33]. The description of splicing variants of *ENTPD5* having contrasting impacts as risk or protection factors will be important for these type of studies since targeting different variants may impact the outcome of treatment depending on tumor type. Additionally, it is important to establish if the activity inhibitors can act on different variants since there are differences predicted to affect the tertiary structure of NTPDase5 [24].

Materials and methods

Identification of splicing variants and protein isoforms

Protein entries in the NCBI Protein database were queried using "ENTPD5" OR "PCPH" as a search term on July 14, 2020. After filtering for human data, 31 items were found. The sequences of the equivalent mRNA transcripts were then aligned to the human *ENTPD5* gene using the BLAST tool. The Ensembl database was also queried for transcript variants of ENTPD5 and complete sequences for 3 protein-coding transcripts were found. PSI (percent spliced-in) data for *ENTPD5* splicing events in human cancers were downloaded from the TCGA SpliceSeq database [34] which uses RNA-Seq data. The Proteomics DB [35] tool was queried for evidence of protein isoforms of NTPDase5 based on publicly available mass spectrometry data.

Gene expression, miRNA target prediction, and survival analyses

Gene expression (RNA-Seq) and clinical data were downloaded from the TCGA Research Network using cBioPortal [36, 37]. Detailed information for the selected studies can be found in Supporting Information 1. miRNA data was downloaded from miRCancerdb [38] and TCGA GDAC Firehose (Broad Institute TCGA Genome Data Analysis Center (2016): Firehose stddata__2016_01_28 run. Broad Institute of MIT and Harvard. DOI doi:10.7908/C11G0KM9). miRNA target prediction was made using miRDB [39]. Cox proportional-hazard was performed using the "survival" package of R (www.r-project.org). Unadjusted models were performed separately for z-score transformed of global expression and of PSI. The adjusted model accounted for clinical variables, as described in Supporting Information 1. Analysis of weighted Schoenfeld residuals was used to test the proportional hazard assumption. Variables that did not respect the proportional hazard assumption were stratified and included if appropriate. All graphs and analyses were made using GraphPad Prism version 6.04 for Windows (La Jolla, CA, USA) unless stated otherwise. Results were considered significant when $p < 0.05$.

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Author contribution R.P.d.C conceived and designed the work, analyzed and interpreted data, and drafted the manuscript. M.R.W and G.L. interpreted data, edited, and revised the manuscript. All authors approved the final version of the manuscript.

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Data Availability Data sharing not applicable to this article as no datasets were generated. The data analyzed during the current study are available in NCBI Protein (www.ncbi.nlm.nih.gov/protein), Ensembl Genome Browser (www.ensembl.org/index.html), cBioPortal (www.cbioportal.org/), GDAC Firehose (www.gdac.broadinstitute.org/), miRDB (www.mirdb.org/), miRCancerdb (www.mahshaaban.shinyapps.io/miRCancerdb/), TCGA SpliceSeq (www.bioinformatics.mdanderson.org/TCGASpliceSeq/), and Proteomics DB (www.proteomicsdb.org/).

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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