Epigenetic Dysregulation of the Expression of *PRSS3* Splice Variants Increases the Heterogeneity of Transcripts and Functionality in Human Hepatocellular Carcinoma

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Abstract

Background

Hepatocellular carcinoma (HCC) is one of the most lethal human tumors with extensive heterogeneity. Serine protease 3 (PRSS3) is an indispensable member of the trypsin family and has been implicated in the pathogenesis of several malignancies including HCC. However, paradoxical effects of *PRSS3* on carcinogenesis impede the utilization of its biomarker potential. We hereby systematically dissected the expression of four known splice variants of *PRSS3* (*PRSS3-SVs*) and their functional relevance to HCC.

Methods

The expression and DNA methylation of *PRSS3* transcripts and their associated clinical relevance in HCC were analyzed using several publicly available datasets and were validated using qPCR-based assays. Functional assays were performed on gain- and loss-of-function cell models, in which *PRSS3* transcript constructs were separately transfected after *PRSS3* expression was knocked out by CRISPR-Cas9 editing.

Results

PRSS3 expression was differentially decreased in HCC cell lines and tissues attributable to aberrant expression of *PRSS3-SVs* towards bipolarity, in which *PRSS3-V2* and then *-V1* were dominantly expressed whereas *PRSS3-V3* and *-V4* were rarely or minimally expressed. The expression of *PRSS3-V2* or *-V1* was reversely associated with site-specific CpG methylation and was distinct between lowly-expressed *PRSS3-SVs* with hypermethylation ($mPRSS3^{Low}$) and highly-expressed *PRSS3-SVs* with hypomethylation ($umPRSS3^{High}$). Function analysis revealed that *PRSS3-V2* exhibited oncogenic functions distinct from a tumor-suppressive role of ectopic *PRSS3-V1* or *-V3*, or *PRSS3-V4* with inhibitory effects in *PRSS3* knockout HCC cells. Clinically, aberrant expression of *PRSS3-SVs* was translated into divergent relevance in patients with HCC, in which significant epigenetic downregulation of *PRSS3-V2* was seen in early HCC and was associated with favorable patient outcome.

Conclusions

Aberrant expression of divergent *PRSS3-SVs* disrupted by CpG methylation may integrate the effects of oncogenic *PRSS3-V2* and tumor-suppressive *PRSS3-V1*, resulting in the molecular diversity and functional plasticity of HCC. Dysregulated expression of *PRSS3-V2* by site-specific CpG methylation may have potential diagnostic value for patients with early HCC.

Background

Human primary liver cancer is one of the most lethal tumors with a dismal prognosis, featuring extensive heterogeneity and aggressiveness in the context of genetic and epigenetic aberrations [1–5]. Regardless of many approaches developed for the management of liver cancer in the past decade, its incidence and mortality rate continue to increase worldwide [1]. Liver hepatocellular carcinoma (HCC or LIHC) accounts for approximately 75–85% of all primary liver cancers. Most HCCs (> 90%) develop from chronic inflammation-induced liver cirrhosis contributed by multiple risk factors such as hepatitis viruses, alcohol consumption, and non-alcoholic fatty liver disease, which trigger the molecular complexity of intratumor heterogeneity (ITH) increasing HCC phenotypic diversity and therapeutic resistance [1, 2].

Large-scale bioinformatics datasets generated with next-generation sequencing technologies reveal a comprehensive landscape of genomic and epigenetic heterogeneity among HCC cell lines and tissue specimens [3–7]. These studies offer invaluable insight into the molecular basis of ITH to categorize HCC into proliferative and non-proliferative subclasses in favor of integrative molecular monitoring malignant transformation and management of HCC. However, aside from most genetic alterations occurred in passenger genes that may be associated with aging and pollution, most genetic variants such as driver mutations in *TP53*, *TERT* and *CTNNB1* detected in HCCs are not clinically relevant, or are not potentially targetable for the existing drugs [2]. This gives rise to a growing drive to integrate non-genetic variations into ITH, and to distinguish between functional and non-functional ITH [7, 8]. Pre-mRNA alternative splicing (AS), as a key co- and post-transcriptional process drives non-genetic phenotypic heterogeneity, disruption of which generates aberrant splice variants (SVs) that contributes to ITH and functional divergence, thus functionally important to carcinogenesis and oncotherapeutics resistance [9–12].

Proteases play critical roles in multiple biological processes and are associated with a wide variety of pathological conditions, including carcinogenesis [13]. As a group of the trypsin-family serine proteases, human trypsinogen genes, protease serine 3 (*PRSS3*), encodes PRSS3, also called mesotrypsinogen (MTG) [14–16]. *PRSS3* gives rise to four experimentally validated SVs, referred to as trypsinogen transcript variant 1, 2, 3, and 4 (*PRSS3-V1*, *-V2*, *-V3* and *-V4*), encoding PRSS3 isoform 1 (also known as brain form or trypsinogen 4, TRY4) [16, 17], PRSS3-2 (isoform form C or MTG) [15, 18], PRSS3-3 (isoform form B or trypsinogen IV) [19], and PRSS3-4 (new isoform form or trypsinogen 5), respectively [20]. Other than PRSS1 and PRSS2 as the major digestive enzymes in the pancreas, PRSS3 is a minor constituent trypsin isoform but physiologically critical due to its resistance to common trypsin inhibitors [13–15]. In addition to digestive activity, PRSS3 has long been implicated in the pathogenesis of several malignancies and is therefore a promising biomarker and potential therapeutic target for cancer [21–31]. However, the functional roles associated with the expression of *PRSS3* in cancer development are debatable. On one hand PRSS3 was shown to be upregulated in association with cancer metastasis, recurrence and poor prognosis [21–26, 28–31]. But on the other hand, *PRSS3* was suggested as a tumor suppressor gene due to epigenetic silencing [32–36]. Although the evidence supports the dual roles of proteases in carcinogenesis depending on cellular sources and cancer microenvironment [11–13, 21–23, 32, 33, 35], the underlying molecular basis of PRSS3 for its pro- and anti-tumorigenic roles shown in different cancer types, even reported in the same type of cancer, such as in esophageal adenocarcinoma

[25, 34], lung cancer [24, 33] and liver cancer [28, 35], remains elusive that caused many miscellaneous aliases to *PRSS3* impact its potential target-therapeutic applications [4, 12, 13, 22, 27, 35].

As SVs emerge as new candidates for diagnostic and prognostic biomarkers and therapeutic targets [10, 11], we systematically investigated the expression and epigenetic alteration of *PRSS3*-SVs functionally in relation to HCC heterogeneity. We found that differential expression of *PRSS3* in HCC was attributed to aberrant expression of divergent *PRSS3*-SVs, which was epigenetically dysregulated by site specific abnormal CpG methylation. We also found different functionality and clinical relevance of *PRSS3*-SVs in HCC cells and tissues. Therefore, epigenetic dysregulation of expression of *PRSS3*-SVs may be the molecular basis of *PRSS3* to exert paradoxical effects on hepatocarcinogenesis.

Materials And Methods

Data availability

The datasets used for this study are publicly available on the websites: the Cancer Model Repository (LIMORE) (<https://www.picb.ac.cn/limore/home>) [6]; the Cancer Genome Atlas (TCGA, <https://www.cancer.gov/>) [37]; the Gene Expression Profiling Interactive Analysis (GEPIA, <http://gepia.cancer-pku.cn/>) [38]; the Cancer Cell Line Encyclopedia (CCLE, <http://www.broadinstitute.org/ccle>) [39]; the Cancer Dependency Map (DepMap, <https://depmap.org/portal/>, DepMap Public 20Q3)[40]; the Broad Genome Data Analysis Center (<http://gdac.broadinstitute.org>) [41].

Cell lines

Human HCC cell lines include well differentiated (HepG2 and Huh7) and poorly differentiated (SK-Hep-1, SMMC-7721 and LM3) cell lines and were authenticated by STR profiling. The origin and growth conditions of all cell lines used in the study were described previously [35, 42]. The cells were split to low density (30% confluence) for overnight culture, and were then treated with 5 μ M of 5-aza-2'-deoxycytidine (5-aza-CR, Sigma-Aldrich, St Louis, MO, USA) for 96 hours with the medium exchanged every 24 hours.

Cell line construction

HepG2 and SK-Hep-1 cells with stably ectopic expression of *PRSS3*-V1, -V2, -V3 and -V4 were constructed as described [35]. CRISPR-Cas9 system was used to generate a Huh7 cell line with knockout *PRSS3* (*PRSS3*^{KO}). *PRSS3*^{KO} Huh7 cell line was then separately transfected *PRSS3*-V1, -V2, -V3 or -V4 construct to establish stable re-expression of *PRSS3* transcripts dubbed *PRSS3*^{KO+V} cell model. The cDNA coding human *PRSS3*-V1 (NM_007343.3), -V2 (NM_002771.3), -V3 (NM_001197097.2) and -V4 (NM_001197098.1) were purchased from GeneCopoeia (Rockville, MD, USA) to construct recombinant plasmid. *In vitro* transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The CRISPR/ Cas9 bivector lentivirus was custom ordered from Genechem (Shanghai, China). The sgRNA was GGCACTGAGTGCCTCATCTC.

Cell viability

HepG2, SK-Hep-1 and Huh7 cells were seeded into 96-well plates at 2×10^3 cells/well. Cell viability was measured everyday by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay Kit (KeyGEN Biotech, Jiangsu, China). The absorbance at 490 nm wavelength was detected using a microplate reader (Thermo Multiskan MK3, Thermo Fisher Scientific Inc., USA).

Colony formation

HCC cells were seeded in 6-well tissue culture plates (100 cells/well) in triplicate. Colonies with more than 50 cells were counted after 2 weeks. The cells were fixed with 75% ethanol for 30 minutes and stained with 0.2% crystal violet (Beyotime Ltd., Jiangsu Province, China) for 20 minutes.

Transwell invasion assay

Transwell apparatus was used with 8- μ m polyethylene terephthalate membrane filters (Corning Inc.; Corning, NY, USA). The upper chambers were seeded with 200 μ l of serum-free medium containing 1×10^4 of serum-starved cells. The lower chambers were filled with 500 μ l of 10% FBS-DMEM. After 24 hours, cells that invaded to the lower chamber were fixed and stained with 0.2% crystal violet (Beyotime) as previously described [35].

RNA isolation and RT-qPCR

Cells were harvested for RNA isolation using RNeasy Mini Kit (QIAGEN) and first strand cDNA was synthesized with the Superscript First-Strand Synthesis System (Invitrogen). RT-qPCR was performed using primers as described [35]. The relative expression level of each mRNA was normalized by β -actin using $2^{-\Delta\Delta C_t}$ method

Methylation-specific qPCR (MS-qPCR)

DNA extraction, bisulfite modification and MSP-PCR were performed as described [35, 43]. Genomic DNA was extracted from tissues using the QIAamp DNA mini Kit (Qiagen) followed by quantitative analysis using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Bisulfite modification of DNA was performed using Zymo DNA Methylation Kit (Zymo Research). The positive and negative template control were the Human Methylated & Non-methylated DNA Set (Zymo Research). MSP-qPCR was performed by using methylated or unmethylated primer pairs specifically for PRSS3 [35] and β -actin [43]. The relative level of methylation and unmethylation of PRSS3 was normalized to β -actin using the $2^{-\Delta\Delta C_q}$ method.

Methylated DNA immunoprecipitation-qPCR (MeDIP-qPCR)

Genomic DNA was extracted from the HCC cells. The purified DNA is then subjected to sonicated into 200~1000bp fragments. A 10% sonicated DNA was kept as input control. The denatured DNA fragments

(Input fractions) were incubated with 2 µg anti-5-methylcytidine (5mC) (Active motif, 91311) or 2 µg control IgG (Sigma-Aldrich, I5006) monoclonal antibodies at 4°C overnight, following by precipitation using protein A beads. After washing, immunoprecipitated DNA (IP fractions) and input control fraction were purified by using a QIA quick purification kit (QIAGEN, Valencia CA, USA) and analyzed by qPCR using primers as following: F: 5'- CTGTGATGGAGAGGGGGTTC -3'; R:5'- GAGTAGTGTGCGCATCGGT-3'.

Statistical analysis

The data are expressed as means ± standard deviation (SD) of at least three independent experiments. *PRSS3* transcripts expression, epigenetic alterations and associative clinicopathological correlation were analyzed by using the two-tailed Student's t-test, Wilcoxon rank sum test, one-way analysis of variance (ANOVA) with Tukey's post hoc test, Spearman rank test and Fisher's exact test, or χ^2 or Fisher's exact tests. Cancer-related survival was analyzed using Kaplan–Meier method, and was compared using log-rank tests. Statistical significance was considered when $P < 0.05$. All statistical analyses were performed using SPSS version 23.0 (IBM Corp.).

Results

Differentially decreased *PRSS3* expression in HCC attributable to disruption of *PRSS3* transcripts

The RNAseq data from the DepMap portal demonstrated differential expression of *PRSS3* in 24 human HCC cell lines (Fig. 1A, **Table S1**). This was further shown in 81 HCC cell lines from the Cancer Model Repository (LIMORE) (Figure S1A). *PRSS3* expression in HCC cell lines determined by RT-qPCR revealed levels of very low (*PRSS3*^{Low}) to very high (*PRSS3*^{High}) as compared to human fetal liver L02 cells (Fig. 1B). Comparative analysis using the TCGA RNAseq data from FIREHOSE Broad GDAC showed divergent features of *PRSS3* expression in HCC tissues compared to their matched non-tumor tissues (n = 50) (Fig. 1C, **Table S2**), which was further evidenced from analysis of more HCC tissue specimens (Tumor = 371) (Fig. 1D, Table 1). GEPIA portal combined TCGA with GTEx RNAseq datasets showed that *PRSS3* expression was more varied in HCC tissues (n = 369) than in normal controls (n = 160) (**Figure S1B**)[37, 41], albeit with no statistical significance. *PRSS3* mRNA level was lower but with an extraordinary wide range in HCC tissues as compared to normal tissues, suggesting an aberrant and differential expression of *PRSS3* expression in HCC.

Table 1
Correlation between the mRNA levels of *PRSS3* transcripts with clinicopathologic characteristics in patients with HCC

Characteristics	N	<i>PRSS3</i> Expression		<i>P</i>
		High	Low	
		N (%)	N (%)	
Total	371	184 (49.6)	187 (50.4)	
Gender				
Male	250	123 (49.2)	127 (50.8)	
Female	121	61 (49.6)	60 (50.4)	0.9137
Cancer stage				
I	171	82 (47.9)	89 (52.1)	
II	86	43 (50.0)	43 (50.0)	0.8591
III	85	46 (54.1)	39 (45.9)	0.4259
IV	5	3 (60.0)	2 (40.0)	0.6736
Undefined	24	10 (41.7)	14 (8.3)	
Tumor grade				
I	55	21 (38.2)	34 (61.8)	
II	177	88 (49.7)	89 (50.3)	0.1794
III	122	67 (54.9)	55 (45.1)	0.0576
IV	12	5 (41.7)	7 (58.3)	1
Undefined	5	3 (60.0)	2 (40.0)	

To explore the molecular basis of the divergent expression of *PRSS3* in HCC, we dissected the expression of four identified *PRSS3*-SVs expressed in various tissues [15–20] (Fig. 2A). Analysis of the DepMap data revealed that in 24 HCC cell lines, *PRSS3*-V1 and -V2 were two major transcripts contributing to the expression of *PRSS3* because *PRSS3*-V3 was poorly expressed while *PRSS3*-V4 was absent (Fig. 2B, **Table S1**). RT-qPCR showed that despite almost undetectable *PRSS3*-V4 and very low expression of *PRSS3*-V3 in all cell lines, *PRSS3*-V1 was expressed at low levels in L02 cells, whereas *PRSS3*-V1 and -V2 were minimally expressed in HepG2, SK-Hep-1 and SMMC-7721 cells but highly expressed in Huh7 and LM3 cells (Fig. 2C).

Through analysis of the expression level of *PRSS3*-SVs in 50 paired tissue samples, we found that *PRSS3*-V2 and also -V1 were predominantly present in both normal and tumor tissues (Fig. 2D, **Table S2**).

Figure 2E showed that the expression of *PRSS3*-SVs was decreased in 371 HCC tissue samples in a bipolar pattern as compared to normal liver tissues. Co-expression analysis of both HCC cell line and tissues summarized in Table 2 further showed that the highest contribution of *PRSS3*-SVs to *PRSS3*^{High} was co-expressed *PRSS3*-V2 and -V1, not by either *PRSS3*-V2 or -V1 in the specimens, confirming *PRSS3*-V2 was the predominant transcript in *PRSS3*^{High} in HCC. Meanwhile, *PRSS3*^{Low} was also resulted from decreased expression of *PRSS3*-V2 and/or -V1 because the minimally expressed *PRSS3*-V3 minimally affected the eventual expression of *PRSS3* despite *PRSS3*-V3^{Low} most frequently associated with *PRSS3*^{Low} in HCC. These results thereby revealed disruption of *PRSS3* transcripts towards a bipolar expression contributing to aberrant and differential expression of *PRSS3* in HCC, in which *PRSS3*-V2 was a dominant transcript leading to *PRSS3* expression.

Table 2
Predominance and co-expression of *PRSS3* transcripts in HCC cell lines and tissues

Transcript(s)	Cell lines (n = 24)				Tissue specimens (n = 371)			
	High	%	Low	%	High	%	Low	%
PRSS3	12	100	12	100.00	184	100.00	187	100.00
PRSS3-V1	9	75.00	9	75.00	163	88.59	166	88.77
PRSS3-V2	10	83.33	9	75.00	173	94.02	175	93.58
PRSS3-V3	1	8.33	11	91.67	48	26.09	185	98.93
PRSS3-V1 + V2	8	66.67	6	50.00	153	83.15	156	83.42
PRSS3-V2 + V3	0	0.00	9	75.00	45	24.46	173	92.51
PRSS3-V1 + V3	1	8.33	9	75.00	46	25.00	165	88.24
PRSS3-V1 + V2 + V3	0	0.00	6	50.00	43	23.37	155	82.89

Aberrant expression of divergent *PRSS3* transcripts in HCC is epigenetically dysregulated by site-specific abnormal CpG methylation.

We next assessed the contribution of DNA methylation to the expression of *PRSS3*-SVs based on the data available from the DepMap and the FIREHOSE [39, 40] for three genomic regions in *PRSS3*. These were referred to as extended promoter region with 17 CpG sites defined as CpG 1–17, upstream (-1000 bp upstream from the TSS of *PRSS3*-V1/V3 covering 2–7 of the 17 CpG sites) and extensive fragment containing 6 CpGs (CpG A-F) (Fig. 3A, **Table S4**). Association analysis demonstrated an inverse association between the upstream methylation and mRNA expression of *PRSS3* and its transcripts *PRSS3*-V1, -V2 that were distinct between hypermethylation of *PRSS3*^{Low} (m*PRSS3*^{Low}) and hypomethylation of *PRSS3*^{High} (um*PRSS3*^{High}) in HCC cell lines (Fig. 3B) and tissues (Fig. 3C).

Given that epigenetic silencing of *PRSS3* has been observed in several types of human cancer [32–35], we investigated DNA methylation in the extended promoter region of *PRSS3* upon the data availability (Fig. 3A). Unsupervised clustering combined with spearman correlation analysis of methylation states and expression of *PRSS3* transcripts in HCC cell lines revealed that among 17 CpGs distributing in the extended promoter region, methylation occurred at CpG site 5–17 (–89 ~ 653 bp from the TSS of *PRSS3-V1/V3*) was reversely correlated with the mRNA expression level of *PRSS3-V1*, while methylation at CpG site 12–16 (522 to 564 bp to *PRSS3-V1* TSS) was highly related to *PRSS3-V2* expression (Fig. 3D, **Figure S2, Table S5**). No associative comparison was conducted on *PRSS3-V3* and *-V4* due to their rare expression in HCC. Moreover, despite a positive association shown in CpG site F, methylation at CpG site A-E was negatively correlated with *PRSS3* expression (Fig. 3E, **Figure S3**). CpG site methylation at the extensive fragment of *PRSS3* was decreased at site A, increased at B, C and D, and then decreased at E and F in HCC tumors as compared to normal controls (Fig. 3F). These data suggest that an extended promoter region was important for epigenetic regulation of *PRSS3* transcripts.

We then examined methylation-specific effect on *PRSS3* expression using qPCR-based assays (Fig. 3A). MSP-qPCR showed hypermethylation in *PRSS3^{Low}* cell lines (L02, HepG2, SK-Hep-1) in contrast to hypomethylation in *PRSS3^{high}* Huh7 cells (Fig. 3G). Figure 3H revealed that treatment with DNA methyltransferase inhibitor 5-aza-CR caused significant upregulation of *PRSS3* expression in *PRSS3^{Low}* cell lines, but had no effect on *PRSS3^{High}* Huh7 cells. Notably, a bipolar expression pattern was observed in *PRSS3^{Low}* cell lines upon 5-aza-CR treatment showing significant upregulation of *PRSS3-V1* and *-V3* opposite to downregulation of *PRSS3-V2*, eventually integrative to the upregulation of *PRSS3*, whereas the treatment had no effect on *PRSS3^{high}* Huh7 cells, actually due to integration between upregulation of *PRSS3-V2* and downregulation of *PRSS3-V1* or *-V3*. MeDIP-qPCR further showed that anti-5-methylcytosine (5-mC) antibody enriched significantly less genomic DNA fragments in HepG2 cells but not in Huh7 cells upon 5-aza-CR treatment (Fig. 3I), suggesting that 5-aza-CR was effective in the expression of *PRSS3* specifically by altering the DNA methylation in this target region. Although the expression of *PRSS3-V3* in L02 or *PRSS3-V2* in HepG2 and SK-Hep-1 cells was too low to be taken into account its decreased significance level, these results consistent with bioinformatic analysis of HCC cell lines and tissues, as well as our previous report [35], suggest that methylation occurring at this region is more critical for epigenetically controlling *PRSS3* transcript activities in HCC. As shown in the summarized table (Fig. 3J), *PRSS3-SVs* were divergently expressed and response to 5-aza-CR treatment associated with site-specific CpG methylation that eventually determined the expression level of *PRSS3* as a whole, suggesting that epigenetic dysregulation of the expression of *PRSS3-SVs* by site-specific CpG methylation may mediate their functional differences in HCC.

PRSS3-V2 exerts oncogenic functions distinct from tumor-suppressive effects of PRSS3-V1 and -V3 in HCC cells

The functional role of *PRSS3-SVs* was assessed by transfecting *PRSS3-V1* to *-V4* respectively into *PRSS3^{Low}* HepG2 and SK-Hep-1 cells (defined as V1 to V4) (Fig. 4A). MTT assays showed that ectopic

expression of *PRSS3-V1* or *-V3* significantly inhibited HCC cell proliferation in contrast to notably enhancing effect by ectopic *PRSS3-V2* expression, or in addition to unfunctional *PRSS3-V4* in HCC cell proliferation as compared to the vector controls (Fig. 4B). Moreover, the results of clone formation assay showed that overexpression of *PRSS3-V1* or *-V3* remarkably diminished the number of colonies of HCC cells compared with the control group, but *PRSS3-V2* overexpression resulted in an increased number of colonies only effectively in HepG2 cells. However, ectopic *PRSS3-V4* significantly reduced clone formation in SK-Hep-1 cells but had no effect in HepG2 cells (Fig. 4C). Transwell assay further showed an inhibitory effect of *PRSS3-V1* or *-V3* on HCC cell migration, opposite to *PRSS3-V2* that showed an enhanced effect in the cells (Fig. 4D). These results suggest a tumor-suppressive effect of *PRSS3-V1/V3* versus an oncogenic effect of *PRSS3-V2* in HCC cells.

To further define the phenotypic properties of *PRSS3-SVs* in HCC cells, we established a *PRSS3^{KO+V}* cell model, in which each *PRSS3* transcript construct was separately transfected after endogenous *PRSS3* was knocked out through CRISPR/Cas9 system (Fig. 5A). RT-qPCR showed that all the detected *PRSS3* transcripts were effectively knocked out and their constructs were stably expressed in Huh7 cells, respectively designated as *PRSS3^{KO+V1}* to *PRSS3^{KO+V4}*, or the vector control (*PRSS3^{KO+C}*) (Fig. 5B). Functional assays as shown in Fig. 5C to 5E revealed that *PRSS3* knockout in Huh7 cells facilitated cell proliferation, colony formation and migration, which were abolished by re-expression of *PRSS3-V1* or *-V3*. Ectopic re-expression of *PRSS3-V2* augmented the *PRSS3*-knockout effects on cell proliferation, colony formation, and remarkably, on migration of *PRSS3^{KO}* Huh7 cells. Unexpectedly, *PRSS3-V4* re-expression did not affect Huh7 cell proliferation but resulted in significantly inhibition of *PRSS3^{KO}* Huh7 cell activity. These results demonstrate dual roles of *PRSS3-SVs* in HCC cells and divergent disruption of *PRSS3* transcripts may be integrated to establish their functional heterogeneity in HCC cells.

Epigenetic alteration of *PRSS3-V2* is associated with clinical relevance in patients with early HCC

To further explore the contribution of *PRSS3* transcripts to tumor heterogeneity, we used TCGA dataset to analyze their clinical relevance. We found that the expression of *PRSS3* and *PRSS3-V2* was similarly downregulated but with a gradually increased tendency in HCC tumors compared with control tissues, following the progression of tumors stages (Fig. 6A) and pathological grades (Fig. 6B), in which *PRSS3-V2^{Low}* was significantly detected in tumors of early HCC patients in contrast to *PRSS3-V2^{High}* in advanced tumors. Kaplan-Meier (K-M) analysis revealed that *PRSS3-V2^{Low}* was a favorable factor for overall survival of HCC patients based on cancer stages (Fig. 6C) and grades (Fig. 6D), in which *PRSS3-V2^{Low}* patient groups with low-grade tumors showed significantly favorable outcome ($P = 0.011$). Moreover, divergent disruption of CpG site methylation (A to F) was shown throughout clinical progression of tumors but occurred more frequently and significantly in tumors of HCC patients with early-stage (Fig. 6E) and lower-grade tumors (Fig. 6F). In such tumors alteration in CpG methylation at site D was most reversely correlated with the expression of *PRSS3* and *PRSS3-V2*. Since the region located at site D was shown as an important regulatory region specifically for epigenetic regulation of *PRSS3* transcripts (Fig. 3), the data suggest that site-specific epigenetic alteration of *PRSS3-V2* in HCC tissues was distinct

between *mPRSS3-V2^{Low}* in early HCC and *umPRSS3^{High}* in advanced HCC patients, in which early HCC patients with *PRSS3-V2^{Low}* tumors had better outcomes.

Discussion

Paradoxical effects of many genes have been observed during tumorigenesis [13, 44, 45]. In this study we explored the dysregulation of splicing variants expression functionally contributing to HCC heterogeneity. Protease PRSS3 is the first to link the enzyme to prostate cancer leading to the development of a compound to stop PRSS3 from promoting metastasis [13, 46]. Since the high similarity in both sequences and structures to different trypsinogen isoenzymes made it difficult to delineate their functionally associated transcripts distributed in different tissues [13, 14], the protumor [21–31] or antitumor properties of PRSS3 [32–36] were deciphered relying on the cellular source and cancer microenvironment [13, 14, 35]. In this study, we found differentially expressed *PRSS3* in HCC due to CpG methylation-mediated epigenetic dysregulation of its splice variants. Different *PRSS3-SVs* expressed in HCC showed a dual role in hepatocarcinogenesis that may increase phenotypic diversity. Our study uncovered an epigenetic-mediated *PRSS3* transcript variance contributing to non-genetic phenotypic diversity of HCC [44]. To our best knowledge, this is the first study of functional dissection of the expression of *PRSS3-SVs* in cancer and thus has important implications in HCC patient-tailored management.

PRSS3 is known as a digestive protease with restricted expression in pancreas. However, the preferential expression of *PRSS3-SVs* differs in human tissues suggests a tissue-selective expression manner. For instance, *PRSS3-V2* was exclusively expressed in human pancreatic tissue and fluid encoding MTG [16, 47]. Canonical *PRSS3-V1* was originally identified in human brain [17, 47]. *PRSS3-V3* shares a same TSS with *PRSS3-V1* but has a different in-frame exon with deduced a 261-amino acid sequence (formerly named isoform B) [19]. *PRSS3-V4* was cloned from keratinocytes and shown in participating keratinocyte terminal differentiation [20]. Our study showed the differential expression of *PRSS3* as a DEG in HCC across a large expression range that could be used to phenotypically distinguish between *PRSS3^{Low}* and *PRSS3^{High}* HCC cells and tissues. Accordingly, we found divergent expression of *PRSS3-SVs* towards bipolarity following clinical progress from downregulation in early HCC to upregulation in advanced cancer, unveiling the molecular basis of *PRSS3* in tissue-selective expression of its splice transcripts in HCC. Despite the infrequent or minimal expression of *PRSS3-V3* and unexpressed *PRSS3-V4*, the divergent expression changes of *PRSS3-V2* and/or *-V1* were major contributors to the transcript heterogeneity of *PRSS3* in HCC. Notably, the expression of *PRSS3-SVs* was dynamically altered following clinical progress from downregulation in early HCC to upregulation in advanced cancer. *PRSS3* transcript heterogeneity was further evidenced by its divergent responses to 5-aza-CR treatment of HCC cells, distinguishing between upregulation of *PRSS3-V1* or *-V3* but downregulation of *PRSS3-V2* in *PRSS3^{Low}* HCC and downregulation of *PRSS3-V1* but upregulation of *PRSS3-V2* in *PRSS3^{High}* HCC. The divergent expression of *PRSS3* transcripts and their responding to 5-aza-CR prompted our consideration of the effects of non-genetic heterogeneity on chemotherapy-response, because this well-known anticancer drug

has broad clinical applications and the mis-splicing regulation as non-genetic mechanisms is frequently linked to therapy escape [48–50]. For precise evaluation of the clinical effectiveness and drug resistance by using a DEG, its functional splice variants, rather than its overall expression, need to be taken into account. Nevertheless, it was clear that differentially expressed *PRSS3* decreased as a whole was mainly attributable to its aberrant transcript variance expressed in HCC.

PRSS3 translocates from chromosome 7q34, the locus of *PRSS1* and *PRSS2*, to chromosome 9p11.2, a region frequently containing alterations [13, 51]. However, frequent genetic variations occurred in *PRSS3* have not yet demonstrated disease-associated *PRSS3* variants (https://www.nextprot.org/entry/NX_P35030/medical). Alternative splicing forms dynamic interactome offering precise therapeutic approaches to correcting cancer-specific defects caused by mis-splicing regulation, in which epigenetics plays an essential role [9, 11, 12, 49, 52–54]. Our previously study showed epigenetic silencing of *PRSS3* in HCC [35], we reasoned epigenetic regulation of *PRSS3*-SVs contributing to non-genetic heterogeneity in HCC. The different TSSs and start codes in *PRSS3* suggest that *PRSS3*, like the majority of protein-coding genes, tends to be regulated by multiple or alternative promoters, the usage of which provides a pre-transcriptional control of gene activity to express its different isoforms in a tissue-specific manner [4, 6, 11, 25]. Here, we found an extended promoter region covering the upstream and the intragenic region of *PRSS3*-V1/V3 and -V4, providing a site-specific way to regulate the expression of *PRSS3*-SVs. Both HCC cells and tissues were phenotypically classified as *mPRSS3*^{Low} and *umPRSS3*^{High} based on CpG methylation in association with expression of *PRSS3* transcripts. Compared to the consistent upstream hypermethylation, site-specific CpG methylation in the intragenic region was found more associated with the expression of *PRSS3*-V1 and V2, suggesting that this extended promoter region played a central role in regulation of both *PRSS3*-V1 and V2. Given that epigenetic promoter alterations can change chromatin accessibility of transcription regulatory elements binding to transcription factors [9, 12, 44, 52, 55–57], the upstream hypermethylation of *PRSS3* may impact tissue-specific *cis*-regulatory modules that may alter transcription activity of *PRSS3*-SVs in HCC. Dynamic disruption of different CpG site methylation within the extended promoter region may affect certain transcriptional regulators or splicing factors occupancy, resulting in an alternation in exon skipping to control the expression of *PRSS3*-V1 or -V3. Meanwhile, site-specific epigenetic control of *PRSS3*-V2 suggests that the extended promoter may be a distal regulatory region in regulation of *PRSS3*-V2 through a very different epigenetic pathway [58]. Consistent with this, epigenetic silencing of *PRSS3* was found in several cancer types and our previous study showed an intragenic DNA methylation within the extended promoter region contributing to *PRSS3* downregulation in HCC [35]. This study was the first to dissect epigenetic heterogeneity in regulation of *PRSS3*-SVs that may provide important implications for understanding epigenetic contributions to the genomic occupancy of transcription factors during transcription, in which many events may appear to be co-spliced with distant events[53, 55–57] .

Many transcript isoforms can exist per gene [9–11], most of which are thought not to be functionally relevant [59]. Recently, comprehensive gain- and loss-of function works had shown the functional importance of SVs in tumor heterogeneity by linking genetic variants to individual's phenotypes [52–54,

60, 61]. *PRSS3* appears to be transcribed differentially to display heterogeneous functions in cancer, in which a dual role or contradictory effects reported might be due to MTG (*PRSS3*-V2) to be functionally regarded as *PRSS3* [13, 14, 21, 22, 24]. We hereby deciphered a functional difference among the *PRSS3* isoforms by using constructed Huh7 cell model. Despite *PRSS3*-V2 /MTG-mediated an oncogenic effect in HCC in line with the pro-malignancy activities of MTG shown in other cancer types [13, 14, 21, 22, 24], *PRSS3*-V1 or -V3 were found as tumor-suppressors in HCC cells, while ectopic *PRSS3*-V4 showed an inhibitory effect on the *PRSS3*^{ko} Huh7 cells. *PRSS3*^{ko} resulting in pro-tumor effects in Huh7 cells suggests a tumor-suppressive role of *PRSS3* played in HCC that was attributed to the co-expressed *PRSS3*-V1 and -V2, the two isoforms with opposite functionality. This is in line with our previous observations [35] and may explain some but not all cases of a similar phenotype with well-differentiated and/or low metastatic potential appearing in either *PRSS3*^{Low} (e.g. HepG2, SK-Hep1 cells) or *PRSS3*^{High} (Huh7 cells) liver cancer cell lines, or a dual role of *PRSS3* contradictorily shown in carcinogenesis. To support this, corresponding clinicopathological analysis of HCC specimens compared to the normal tissue controls uncovered that *PRSS3*-V1 and -V2 were main functional components of clinical relevance since *PRSS3*-V1 and -V2 were bipolarly presented in both *PRSS3*^{Low} or *PRSS3*^{High} tissues, thereby their abnormal co-expression could bring out functional heterogeneity including insignificant or paradoxical clinical association. However, a signature pattern of epigenetic regulation of *PRSS3* expression by site-specific CpG methylation showed dynamically from m*PRSS3*^{Low} to um*PRSS3*^{High} through clinical progression, better matched to *PRSS3*-V2, suggesting *PRSS3*-V2 to be a more prevalent isoform functionally through clinical progression of HCC. Accordingly, significant epigenetic downregulation of *PRSS3*-V2 was seen in early HCC with favorable patient outcome. This supports an oncogenic role of *PRSS3*-V2/MTG dominantly in HCC thus providing early diagnostic and prognostic value for HCC [14, 21, 22, 24]. Thus, our study provides additional evidence for supporting the hypothesis of functional hepato-heterogeneity attributed to genetic and epigenetic factors [3–6].

Aberrant expression of *SVs* in cancer generates tumor functional heterogeneity conducting eventual cellular phenotype(s) or influence cell fate determination [4, 5, 7, 8]. In this regard, delineation of the heterogeneity of *PRSS3* expression and epigenetic regulation is critical for clarifying the molecular basis of *PRSS3* transcripts thus facilitating functional interpretation of the paradoxical effects *PRSS3* exerting in cancer development. Functional classification and experimental dissection of *PRSS3*-*SVs* and their response to 5-aza-CR treatment distinct between *PRSS3*^{Low} and *PRSS3*^{High} HCC cells (such as Huh7 *versus* HepG2 cells) may be used as an experimental model for studying *PRSS3* splicing-mediated functional heterogeneity during hepatocarcinogenesis. In contrast to permanent genetic mutations, epigenetic disruptions frequently occurred in early clinical stages and plays an important role in modulating cell malignancy in a progressive and reversible manner. Therefore, delineation of the precise molecular mechanisms underlying epigenetic regulation of *PRSS3*-*SVs* could contribute to molecular phenotypes of HCC.

This study on bioinformatic analysis of RNA sequencing data of *PRSS3*-*SVs* and their clinical relevance gave lots of insignificantly divergent results. For instance, *PRSS3*^{Low} was shown in 50 paired HCC tissues,

consistent with our previous observation [35] and the analyses showing in the TCGA and the UALCAN portal [37]. But its decrease was no longer statistically significant in more HCC tissue specimens, due to different statistical methods, or integration of the RNAseq data with different median cutoff values for extensively divergent expression of *PRSS3-SVs* in HCC specimens. Therefore, the conventional parameter such as the median cutoff values may need to be reevaluated for grouping a DEG with divergent expression levels. Moreover, the functional heterogeneity could be caused by microenvironment enhanced co-expression diversity of *PRSS3-SVs*. As a result, further studies with larger sample size of paired HCC specimens are warranted to validate our observations.

Conclusions

In summary, *PRSS3* was aberrantly expressed in HCC due to epigenetic dysregulation that was integrated with divergent expression of *PRSS3-SVs* by site-specific CpG methylation. The effects of oncogenic *PRSS3-V2* and tumor-suppressive *PRSS3-V1* in HCC cells may increase the molecular diversity and functional plasticity of hepatocarcinogenesis. Epigenetic dysregulation of *PRSS3-V2* distinct between *mPRSS3-V2^{Low}* in early clinical stages and *umPRSS3^{High}* in advanced tumors has potential diagnostic value for patients with early HCC.

Abbreviations

5-Aza-CR 5-aza-2'-deoxycytidine

CCLE the Cancer Cell Line Encyclopedia

DepMap the Cancer Dependency Map

FPKM Fragments Per Kilobase Million

GTEx the Genotype-Tissue Expression

GEPIA the Gene Expression Profiling Interactive Analysis

HCC Hepatocellular carcinoma

LIHC Liver hepatocellular carcinoma

mPRSS3^{Low} lowly-expressed *PRSS3-SVs* with hypermethylation

MTG Trypsin-3 isoform 2/mesotrypsinogen;

PRSS3 Serine protease 3 gene

PRSS3 Serine protease 3

PRSS3High or PRSS3Low highly- or lowly- expressed PRSS3

PRSS3-V1~-V4 PRSS3 transcript variant 1, -2, -3 and - 4

PRSS3-SVs splice transcripts of PRSS3

TCGA The Cancer Genome Atlas

TPM Transcripts Per Million

TRY4 trypsin-3 isoform 1/trypsinogen 4

umPRSS3High highly-expressed PRSS3-SVs with hypomethylation

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: All authors read and confirmed that this work can be published.

Availability of data and materials: All data are publicly released from TCGA, GTEX, GDAC, GEPIA, FIREHOSE and CCLE databases and hyper-links including citations have been included in the "Materials and Methods" and "Result" section.

Competing interests: The authors declare that they have no competing interests.

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Figures

Figure 1

PRSS3 expression in human HCC cell lines and tissues. (A) The mRNA level of PRSS3 expression in 24 HCC cell lines using RNAseq data extracted from the DepMap website. The expression bar chart is sorted by PRSS3 mRNA expression levels processed as log₂ (TPM+1) scale. TPM: Transcripts Per Million. (B) RT-qPCR analysis of PRSS3 expression in 14 HCC cell lines and human fetal liver cell line L02. The relative expression of PRSS3 mRNA was normalized with β -actin presented as the mean $\pm$ SD from three independent experiments done in triplicate. (C-D) The RNAseq data was sourced from the FIREHOSE (Table 1). PRSS3 expression in 50 pairs of tumors and matched solid normal tissues from patients with HCC (C), or extensively in 371 tumor samples versus 50 normal tissues from HCC patients (D). * $P < 0.05$, by Student t test.

Figure 2

The expression of PRSS3 splice variants in human HCC cells and tissues. (A) A schematic overview of the human PRSS3 gene structures and its splicing transcript variants, and the RT-qPCR primers designed. The top diagram represents the genomic organization of the PRSS3 gene. Alternative splicing within the 5' region of PRSS3 gene leads to the creation of PRSS3-V1 ~ -V4. The exons and introns are represented as boxes and lines (not drawn to scale). E1-E8: Exons; J1-J8: Junctions. E5-8: gray boxes common to all four variants. E1 to 4: sequence-specific for PRSS3-V1 (brown), -V4 (blue), -V3 (purple), and -V2 (green), respectively. Arrowheads indicate primer sets locations used for amplification of PRSS3-SVs. Forward primers were designed specifically for PRSS3-SVs. Reverse primer was common to all. Vc: RT-qPCR primer set common to PRSS3-SVs. (B) Expression level of PRSS3-SVs in HCC cell lines. Data from the DepMap (Table S1). (C) RT-qPCR of PRSS3 transcripts expressed in human fetal liver cell L02 and HCC cell lines. The relative expression level of each mRNA was normalized against β -Actin. (D) Comparison of the mRNA expression of PRSS3 and its transcript variants in 50 paired HCC and normal liver tissues (Table S2). The relative percentage of PRSS3 transcripts expressed in each paired sample (TPM scale) was visualized by 100% stacked bar graph. (E) The mRNA expression of PRSS3 transcripts in HCC tissues (n=371) and normal liver tissues (n=50) based on data from the FIREHOSE. The relative transcript level was presented as log2 (TPM+1) scale. * $P < 0.05$ by Wilcoxon rank sum test.

Figure 3

CpG methylation in regulation of the expression of PRSS3 transcripts in HCC. (A) Schematic of PRSS3 5'-genomic region including extended promoter region, upstream and intragenic fragment. The promoter region (-1749 to 653 bp) shared by PRSS3-V1/3 contains 17 CpG sites (CpGs), including 5 CpGs (CpG site 2-7) in the 1kb upstream fragment (-1000 bp from the TSS of PRSS3-V1/3). The extensive fragment includes 6 CpGs (defined as A, B, C, D, E and F) scattering around a broad genomic region about 34.5 kb scale from -170 down to 34,654 bp of the PRSS3-V1/3 TSS but still -10,643 bp away to the TSS of PRSS3-V2. The genomic position of each CpG site is shown relatively to the TSS of PRSS3-V1/3 (Table S4). Primer-covered regions for MSP-qPCR and MeDIP-qPCR are shown. (B) 1kb upstream methylation normalized as percentage relative to PRSS3 expression in HCC cell lines visualized by 100% stacked bar graph. (C) Spearman and Pearson correlation analysis of 1kb upstream methylation associated with PRSS3 expression in human primary liver tumor samples (n=371). (D) and (E) Clustered heatmap of correlation between CpG site methylation and PRSS3 transcripts expression. Data was visualized by using correlation as distance function for heatmap cluster analysis of the promoter CpG methylation in 20 HCC cell lines (D) and the intragenic 6 CpGs methylation in HCC tissue specimens (n=414) (E). In the

heatmap blue color indicates low, green intermediate and yellow high DNA methylation or mRNA values. Rows: CpGs along correlatively with mRNA expression level of PRSS3 transcripts which were renormalized with mean = 0, standard deviation =1 in accordance with DNA methylation values. Columns: HCC cell lines or tissue specimens. The statistical significance of correlation coefficients between CpG sites (red) and mRNA expression of PRSS3 transcripts were shown at the bottoms. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Figure S3, S4 and Table S5). (F) Association analysis of CpG Site methylation with PRSS3-SVs expression in 414 HCC tissue specimens compared with 41 normal controls (Wilcoxon rank sum test). (G) MS-qPCR of PRSS3 methylation in HCC cell lines and L02 cells. In vitro methylated DNA (IVD) and normal human peripheral lymphocyte DNA (NL) served as positive and negative methylation controls, respectively. (H) RT-qPCR of expression of PRSS3 transcripts in HCC cell lines and L02 cells upon treatment with epigenetic reagents, 5-aza-2'-deoxycytidine (5-Aza-CR: 5 μ M, 96 h). * $P < 0.05$, ** $P < 0.01$ by student's t test. (I) MeDIP-qPCR to analyze 5-mC-enriched genomic fragments associated with the intragenic CpG island (iCpGI) in HCC cell lines and L02 cells after 5-Aza-CR treatment. ** $P < 0.01$ by student's t test. (J) In the summary table, the differential expression changes of PRSS3 transcripts responding to treatment with 5-aza-CR was visualized with symbols and colors. The iCpGI methylation was defined as partial methylation (PM) or methylation (M) based on the MSP results. PRSS3 expression: "-", $< 0.001\%$; "±", $0.001-0.05\%$; "+", $> 0.05\%$; "+++", $> 1\%$. The fold changes upon 5-aza-CR treatment were shown in color: yellow, upregulation; green, downregulation; blue, no change.

Figure 4

Effects of ectopically expressed PRSS3 transcripts on HCC cell malignancy. The PRSS3 splicing variants were separately transfected into HepG2 and SK-Hep-1 cells for establishment of the stable cell lines with individual overexpression of either PRSS3-V1 to -V4 (V1 to V4) or vector control (Control). (A) The mRNA expression levels of PRSS3 transcripts in the transfected cells were measured by RT-qPCR and quantified relative to the control cells (Student t test). (B) Cell viability of HepG2 and SK-Hep-1 cells with ectopic expression of either PRSS3 transcript was detected by MTT assays in comparison with the vector control (two tailed Student's t test). (C) Colony assays showing colony formation of HepG2 and SK-Hep-1 cells after overgrowing for 2 weeks. Representative images were presented on the left panel; quantitation of the colony numbers was shown on the right. (D) Transwell invasion assay assessing cell invasion capacity following transfection of PRSS3 transcripts. Left panel: representative image; Right panel: quantitation of the migrated cells. Scale bar: 50 μ m. One-way ANOVA with Tukey's post hoc test was calculated for the transfected cells compared with the vector control in (C-D). * $P < 0.05$, ** $P < 0.01$, versus

control. Data are presented as mean $\pm$ SD of a representative of three independent experiments done in triplicate.

Figure 5

Functional divergence of PRSS3 transcript variants in a gain- and loss-of function cell model. (A) Schematic of workflow for construction of a cell model by endogenous knockout and then ectopic expression of PRSS3 transcript in Huh7 cells (PRSS3 KO+V cell model). Genomic deletion of PRSS3 transcripts by targeting common exon 5-8 region in PRSS3^{High} Huh7 cells was performed using CRISPR/Cas9 system, followed by transfecting with PRSS3-V1 to -V4 constructs (PRSS3 KO+V1 to PRSS3KO+V4) or vector control (PRSS3KO+C), respectively. Puromycin (Puro) and Geneticin (G418) were used for selection of the transduced cells. (B) RT-qPCR analysis of PRSS3 mRNA expression in the

transfected cells. The relative mRNA expression of PRSS3 transcripts normalized with β -actin (Student t-test). (C) MTT assays showed the viability of Huh7 cells (two tailed Student's t test). (D) Colony formation of Huh7 cells for 2 weeks. Left panel: representative image; Right panel: The colony numbers counted. (E) Transwell invasion assay assessing the invasion capacity of Huh7 cells upon the transfection. Left panel: representative images; Right panel: quantitation of the invaded cells. Scale bar: 50 μ M. One-way ANOVA with Tukey's post hoc test was calculated for the transfected cells compared with the vector control in (C-D). * $P < 0.01$ ** $P < 0.01$, versus control. Data is presented as mean $\pm$ SD of a representative of three independent experiments done in triplicate.

Figure 6

Clinical relevance in HCC patients in association with epigenetic alteration of PRSS3-SVs. (A and B) Box-and-Whisker plot with overlay of individual data points showing mRNA expression of PRSS3 transcripts in HCC tissues (Tumor = 371) and normal controls (Normal = 50), based on cancer stages (171 stage I, 86 stage II, 85 stage III, 5 stage IV) and tumor grades (55 grade I, 177 grade II, 122 grade III, 12 grade IV) (Table 1). (A) PRSS3; (B) PRSS3-SVs. (C and D) HCC patients were group into PRSS3-V2High and PRSS3-

V2Lowgroups based on the mean value of each transcript in tumors (Table 1). The Kaplan–Meier method was used to determine the patient survival and log-rank (Mantel-Cox) test to compare survival rate. Results of HCC patients' survival curves from left to right panels: cancer stages I-II and III-IV (C), tumor grades I-II and tumor grades III-IV(D), respectively. The association of methylation of CpG site A-F within the extended fragment with different clinical stages (E) and pathological grades (F) in HCC tissue specimens (n=414) in comparison with normal liver control tissues (Normal=41). The data was extracted from the FIREHOSE. Statistical significance was determined by Wilcoxon rank sum test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Figure 7

A schematic model for epigenetic dysregulation of PPRSS3 transcripts functionally contributing to hepatocarcinogenesis and its biomarker potential. (A) Epigenetic silencing of PRSS3-SVs by site-specific CpG methylation in tumor of patients with early HCC, in which mPRSS3Low was a potential biomarker favorable for patients' survival. (B) Epigenetic disruption resulted in umPRSS3High in tumor of advanced HCC patients.

Supplementary Files

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