Increased expression of PSME2 is associated with clear cell renal cell carcinoma invasion by regulating BNIP3-mediated autophagy

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Abstract. Previous studies have showed that proteasome activator complex subunit 2 (PSME2) may play a role in some types of cancer. However, the involvement of PSME2 in clear cell renal cell carcinoma (ccRCC) remains unknown. The aim of the present study was to assess the poorly understood function of PSME2 expression in renal carcinoma. Using bioinformatics analysis, PSME2 mRNA expression profiles were investigated, along with its potential prognostic value and its functional enrichment. Signaling pathways and putative hub genes associated with PSME2 in ccRCC were identified. Based on the bioinformatics analysis results, immunohistochemistry of human ccRCC samples and renal carcinoma cell lines (CAKI-1 and 786-O) transfected with short interfering RNA targeting PSME2 were analyzed using western blot analysis, reverse transcription-quantitative PCR, immunofluorescence, and Cell Counting Kit-8, Transwell and transmission electron microscope assays. The results showed that when PSME2 expression was knocked down, the invasive abilities of the tumor cell lines were reduced, while autophagy was enhanced. The present study demonstrated that PSME2 was associated with the invasion ability of ccRCC cell lines by inhibiting BNIP3-mediated autophagy. In summary, PSME2 could be used as a prognostic factor and a promising therapeutic target in ccRCC.

Introduction

The proteasome, also known as a core particle (CP) or 20S proteasome, is a large, major intracellular, multi-catalytic protease in eukaryotic cells (1). The proteasome is responsible for the degradation of most cellular proteins via non-lysosomal proteolytic pathways (2-4). There are four different proteasome regulatory complexes (PA700, PA28, PA28γ and PA200), which affect proteasomal activity (5). PA700, also termed the regulatory complex or 19S, couples with the 20S proteasome, comprising of two distinct but homologous proteolytic pathways (2-4). There are four different proteasome regulatory complexes (PA700, PA28, PA28γ and PA200), which affect proteasomal activity (5). PA700, also termed the regulatory complex or 19S, couples with the 20S proteasome to form the 26S proteasome (5). The 26S proteasome is responsible for breaking down ubiquitinated protein substrates in an ATP-dependent manner (6). PA28, an 11S protein regulator of the 20S proteasome, comprises of two distinct but homologous polypeptides, termed PA28α and PA28β. PA28α and PA28β are encoded by small diverse genes, proteasome activator complex subunit (PSME)1 and PSME2, respectively (7-9). PA28αβ activates the proteasome by binding to the cylinder end of the 20S proteasome and opening the catalytic center (10-12). PA28γ, encoded by PSME3, forms a homoheptamer and binds to the α-ring of the CP (13). PA200 is encoded by PSME4 and, similarly to PA28, binds to the end of the 20S CP (14,15).

PA28αβ is expressed primarily in the cytoplasm, whereas PA28γ and PA200 are highly abundant within the nucleus (16,17). PA28α and β subunits are highly inducible by IFN-γ, and are abundant in the liver, lung and spleen, but has limited expression in the brain. By contrast, PA28γ is not induced by IFN-γ, and is abundant in the brain and has moderate expression in other organs, such as the spleen, testis, and sperm (19). PA28 modulates the proteasome-catalyzed products of the major histocompatibility class I antigenic peptides to present to cytotoxic T lymphocytes, inferring its association with the immune response (20-22). Previous studies have found that PA28 could activate the hydrolysis of small non-ubiquitinated peptides and has protective functions against oxidative stress (23-25). A previous study demonstrated that PA28 was associated with colon cancer, while other studies reported that PA28 plays a role in some cancers (26,27). For example, using two-dimensional polyacrylamide gel electrophoresis-based proteomics, Ebert et al (28) showed that human PA28β protein expression was increased in gastric cancer. Perroud et al (29)

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also discovered that the protein expression levels of PA28β were upregulated in clear cell renal cell carcinoma (ccRCC) compared with that in normal kidney tissue, as determined using proteomics. The mRNA expression levels of PA28β were markedly elevated in cutaneous skin melanoma and Burkitt lymphoma (14,20). Higher protein expression levels of PA28β were detected in primary breast tumors compared with that in lymph node metastasis (30). However, Huang et al (31,32) and Zheng et al (33) showed that the protein expression level of PA28β was decreased in gastric adenocarcinoma (GA) and this decreased expression was associated with poorer GA differentiation. Kim et al (34) confirmed that PA28β expression was decreased in lung cancer. In human esophageal squamous cell carcinoma, PA28β expression was significantly lower (35). In conclusion, PA28β expression is differently associated with several types of cancer.

The present study aimed to investigate PSME2, as a biomarker for ccRCC using the protein expression patterns of kidney renal clear cell carcinoma (KIRC) tissues and matched adjacent normal tissue. In addition, the association between PSME2 expression level and BCL2 interacting protein (BNIP) 3-mediated autophagy in ccRCC was also analyzed. The findings suggest that PSME2 may be a candidate target for kidney cancer therapy.

Materials and methods

Samples and data processing. The mRNA expression data of tumor and normal adjacent tissue from patients with KIRC were downloaded from two platforms, including KIRC cohort from TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). TCGA database comprises of a large number of gene expression data and is a useful resource for understanding the molecular basis of cancer. In the present study, a dataset containing 533 tumor samples and 72 paired normal samples was downloaded. A paired t-test was used to compare the expression level of four PSME genes between the tumor tissues and adjacent normal tissues. The Oncomine database, which is a publicly accessible online cancer microarray database (https://www.oncomine.org/resource/login.html) (36), was used to analyze the mRNA expression level of PSME2 in tumor (n=486) and normal (n=338) tissues from different types of cancer. During the analysis, the numbers in the boxes indicate the number of GEO datasets containing differential expressed PSME2; the red color indicates the expression of PSME2 ranked in top 10% of all genes and blue color indicates PSME2 ranked in bottom 10% of all genes. Various threshold parameters were set as follows: P<0.01; fold change ≥2; gene ranking, all and data type, mRNA. The RNA Sequencing dataset, from the two datasets were expressed as fragments per kilobase per million mapped reads and were log2 transformed after the addition of one [log2(x+1)] prior to analysis.

Survival analysis. Kaplan-Meier plotter was used to calculate the survival time in patients with KIRC in each dataset. The survival probability, including overall survival (OS), disease-specific survival (DSS), and progression-free survival (PFS) times, were evaluated for patients with high or low mRNA expression levels of PSME2, based on the best group separation. The log-rank test was used and P<0.05 was considered to indicate a statistically significant difference. Survival analyses were performed using R software v3.6.3 (https://www.r-project.org/).

Bioinformatics analysis. The top 25% of genes with the largest variance were used to perform a weighted gene co-expression network analysis (WGCNA) with the ‘WGCNA’ package in R software (37). The correlation coefficient and P-value of the module characteristic genes with tumor and normal tissues were calculated using Pearson correlation coefficient (PCC) algorithm. The absolute value of the correlation coefficient was ≥0.5, and P<0.05 was used to screen the modules associated with each trait. Gene dendrogram construction and module identification was performed with the dynamic shear method (38). Further study of the brown module with the tumor tissue, which was significantly associated with PSME2 expression level, was performed for functional enrichment analyses.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to identify the biological functions in the co-expression module. The Database for Annotation, Visualization and Integrated Discovery (DAVID; version 6.8) (39,40) database is a comprehensive set of functional annotation tools for high-throughput gene function enrichment analysis. The biological processes and enriched pathways of the proteins encoded by the candidate genes were analyzed using DAVID. P<0.05 was set as the cut-off criterion.

Gene Set Enrichment Analysis (GSEA; https://www.gsea-msigdb.org/gsea/index.jsp) was performed to screen the GO terms and KEGG pathways that may be associated with PSME2 based on the 6,290 curated gene datasets in the database. In this analysis, all of the ccRCC and normal samples were analyzed and a total of 1,097 kidney cancer samples were divided into two groups based on the expression level of PSME2; low expression level was defined as log₂(TPM+1) <6.2, while high expression level was defined as log₂(TPM+1) >6.2. The cut-off values for GSEA were false discovery rate (FDR) <0.25 and P<0.05.

Bioinformatics analyses were performed using the R programming language (https://www.r-project.org). Cox proportional hazard regression analysis was used to evaluate the association between the risk score and OS, DSS and PFS times. Receiver operating characteristic (ROC) analysis was used to determine the specificity and sensitivity of PSME mRNA expression level in the KIRC tissues. The area under the curve (AUC) was used as an indicator of reliability.

Tissue microarray (TMA) and IHC. The human ccRCC TMA (cat. no. HKidE150CS01) was purchased from Shanghai Xinchao Biological Technology Co., Ltd. The TMA contained 150 tumors and matched adjacent normal kidney tissues (0.5-2.0 cm) (41) from 75 patients, including clear cell carcinoma, chromophobe cell carcinoma, papillary carcinoma and urothelial carcinoma. The clinical variables, including year of diagnosis, age at diagnosis, survival status and sex were collected, in an unbiased manner. These tissues were taken during renal cell carcinoma surgical resection between July 2006 and February 2007, and the last follow-up day was in August 2015. Kidney function and tumor stages were assessed according to the 2010 International Union Against Cancer Tumor-Node-Metastasis (TNM) classification system (42), and curative resection was defined as previously described (43). The following inclusion criteria were used: Only one tumor lesion,
absence of any metastasis and aged 18-70 years. The following exclusion criteria were used: Liver or kidney function insufficient [includes one of the following: Alanine aminotransferase (ALT) < 7.0 or > 40.0 U/l; aspartate aminotransferase (AST) < 13.0 or > 35.0 U/l; ALT/AST > 1; alkaline phosphatase (ALP) < 40.0 or > 110.0 U/l; serum creatinine > 133 µmol/l; urine pH < 5.0 or > 8.0; urine protein > 1.5 g/24 h; or difficult to follow up. Clinicopathological data of the patients with RCC were collected from medical records at The West China Hospital (Sichuan, China). Survival information was obtained from the Social Security Death Index, telephone interviews and medical records, and all pathological data were originally assessed by two pathologists. The present study was performed in accordance with medical ethics and was approved by the West China Hospital of Sichuan University Biomedical Research Ethics Committee (Sichuan, China). Written informed consent was obtained from all participants before collection of the specimens. The IHC staining was performed according to the manufacturer’s protocol using the primary antibody against PSME2 (cat. no. ab183727; 1:150 dilution; Abcam), as previously described (44). The omission of primary antibodies was used as the negative control, and a brown color was considered as positive staining.

Cell culture and transfection. The HK-2 cell line was purchased from the China Center for Type Culture Collection from Wuhan University (Hubei, China) and was cultured in DMEM/F12 supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.). The 786-O, CAKI-1, and A498 cell lines were purchased from the State Key Laboratory of Biotherapy of Sichuan University (Sichuan, China). The 786-O cell line was cultured with RPMI-1640 medium, containing 10% FBS, while the CAKI-1 cell line was cultured in McCoy’s 5a (Modified) medium supplemented with 10% FBS and the ACHN and A498 cell lines were cultured in EMEM, containing 10% FBS, while the CAKI-1 cell line was maintained at 37˚C in a humidified incubator with 5% CO₂.

The small interfering (si)RNA negative control (scrambled NC) (sense, 5'-UUCUCCGAACGUUGUCACGUU-3' and antisense, 5'-ACGUGACACGUUUCAGAATT-3'), siPSME2#1 (sense, 5'-CCAGGAUAUGAGAUGGAAC-3' and antisense, 5'-UUCUCAUCUAUCUCUUGG-3'), siPSME2#2 (sense, 5'-CAGAGAUCUAGCGACGATT-3'), siPSME2#3 (sense, 5'-UUCUCCGAACGUUGUCACGUU-3' and antisense, 5'-CCAGGAUAUGAGAUGGAAC-3'), siPSME2#4 (sense, 5'-CAAGAUUGAAGAUGGAAC-3' and antisense, 5'-UUCUCAUCUAUCUCUUGG-3'), and siBNIP3 (sense, 5'-UACUCUGCGAGCAGCAGCAGCCGAGCTGG-3') and antisense, 5'-UGCAUGGGUCGCCAGACGAdTdT-3') were purchased from Shanghai GenePharma Co., Ltd. For RNA interference, the CAKI-1 and 786-O cell lines were transfected with siNC or siPSME2 and/or siBNIP3 (50 nM) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection at 37˚C in 5% a humidified incubator with CO₂ for 12, 24 and 48 h, the cells were selected with 5 µg/ml puromycin (Sigma-Aldrich; Merck KGaA) after 3-5 days. Puromycin-resistant clones were tested for their ability to overexpress BNIP3 and used for subsequent experimentation.

Transwell invasion assay. The cell invasion assays were performed using a Transwell chamber (24-well; 8 µm pore size; Corning, Inc.). Matrigel matrix on ice (Corning, Inc.) was diluted 1:8 with pre-cooled medium and coated on the upper chamber surface of the Transwell membrane for 0.5-1 h at room temperature. The CAKI-1 and 786-O cell lines stably transfected with siPSME2 and/or siBNIP3 were trypsinized, centrifuged at 300 x g for 5 min at room temperature, and resuspended with serum-free McCoy’s 5a (Modified) medium and RPMI-1640, respectively. The CAKI-1 and 786-O cells transfected with siPSME2 were then treated with bafilomycin A1 (Baf-A1; 1 µmol/l; Selleckchem) for 24 h. The CAKI-1 cell line was transfected with both siPSME2 + siBNIP3 for 48 h. A total of 1x10⁵ cells (200 µl) were added to the upper chamber and 600 µl same medium with 10% FBS, was placed in the lower chamber in each well of a 24-well plate. After incubation at 37˚C for 48 h, the Transwell membrane was removed and a wet cotton swab was used to clear the cells from the upper chamber. Then, the cells that had migrated through the membrane were washed with 0.01 M PBS and fixed with 4% paraformaldehyde at room temperature for 20 min. Next, the cells were stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 10 min and images were captured using a phase contrast microscope (Nikon Corporation).

Cell proliferation assay. The CAKI-1 and 786-O cell lines transfected with siPSME2#1/siPSME2#2 or siNC, were seeded onto a 96-well plate, at a density of 3.5 x10⁴ cells/ml. The cell number was measured every 24 h using a CCK-8 assay (MedChemExpress). Following incubation for 1.5 h at 37˚C in a humidified incubator with 5% CO₂, the absorbance was measured at 450 nm using an automatic microplate spectrophotometer (Thermo Fisher Scientific, Inc.). The experiments were repeated at least three times.

Immunofluorescence (IF) assays. In total, ~1x10⁵ CAKI-1 and 786-O cell lines transfected with siPSME2#2 or siNC were plated on coverslips, cultured overnight at 37˚C, then transfected with green fluorescent protein- microtubule-associated protein light chain3 plasmid (pGFP-LC3; Invitrogen; Thermo Fisher Scientific, Inc.) at a concentration of 1,000 virus particles/cell using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature. After transfection for 24 h, the coverslips were fixed with 4% pro-cooled paraformaldehyde for 20 min at room temperature, then briefly incubated with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 5 min in the dark. Finally, the slides were sealed with neutral balsam and viewed using a confocal fluorescence microscope (Axiovert 200 M; Zeiss GmbH). DAPI was used to label nuclei (blue), and autophagosomes were defined as GFP-LC3 puncta. The fluorescent puncta were analyzed using ImageJ v1.47 software (https://imagej.net).
Transmission electron microscope (TEM). Following transfection with BNIP3 overexpression plasmid, the CAKI-1 and 786-O cell lines were fixed with 2.5% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.4) at room temperature for 1 h. Then, the cells were washed with PBS and fixed with 0.25% neutral glutaraldehyde overnight at 4°C for TEM sectioning. Subsequently, the sections were analyzed using a TEM (JEOL, Ltd.).

Western blot analysis. The cells, transfected with PSME2 siRNAs and BNIP3 overexpression plasmid, were harvested and lysed with RIPA (Beyotime Institute of Biotechnology). The protein concentration of each sample was measured using a Pierce™ Rapid Gold BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) based on the manufacturer's guidelines. Total protein (40 µg) was separated using 12.5% SDS-PAGE, transferred to PVDF membranes (MilliporeSigma), blocked with 5% skimmed milk at room temperature for 2 h, then incubated with the following primary antibodies: BNIP3 (cat. no. ab109362; 1:1,000 dilution; Abcam), PSME2 (cat. no. ab183727; 1:1,000 dilution; Abcam), LC3-I/II (cat. no. ABC929; 1:500 dilution; Sigma-Aldrich; Merck KgaA) sequestosome 1 (SQSTM1; cat. no. 18420-1-AP; 1:1,000 dilution; ProteinTech Group, Inc.) and GAPDH (cat. no. 60004-1-Ig; 1:10,000 dilution; ProteinTech Group, Inc.) on a shaker overnight at 4°C. Following which, the membranes were washed with TBS containing 0.1% Tween-20 three times and incubated with HRP-conjugated secondary antibodies (1:10,000 dilution; ProteinTech Group, Inc.) for 1 h at room temperature. The blotted proteins were observed using Immobilon ECL Ultra Western HRP Substrate (Merck KGaA), scanned with a Chemi-Doc System (Bio-Rad Laboratories, Inc.) and analyzed using ImageJ software (https://imagej.net).

RT-qPCR and gene expression analysis. Following transfection with siPSME2 or BNIP3 overexpressing plasmid for 48 h in the 786-O and CAKI-1 cell lines, total RNA was extracted from the cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). Then, total RNA (1 µg) was reverse transcribed at 37°C for 15 min, then at 85°C for 5 sec using a PrimeScript™ RT reagent kit, with gDNA Eraser (cat no. RR047A; Takara Bio Inc.). qPCR was performed using TB Green® Premix Ex Taq™ II (cat. no. RR820A; Takara Bio Inc.) according to the manufacturer's instructions on a LightCycler96 thermo cycler (Bio-Rad Laboratories, Inc.). The primer sequences were designed by TsingKe Biological Technology and the sequences are as follows: PSME2 forward, 5'-CTTTTTCGGAGGCTGAGGAT-3' and reverse, 5'-AGGGAAGTCAAGTCAGCCAC-3'; GAPDH forward, 5'-GGTTGGCTCTCTGACATTCACAG-3' and reverse, 5'-GGTTGTGGTAGCCAAATTCGGTGT-3'; IL6 forward, 5'-UCCCCTAGTGGTGTGCGC-3' and reverse, 5'-GAAAAGAGCCTAGGGTTC-3'; TNF forward, 5'-CATCCAACCTTCCAGACGC-3' and reverse, 5'-CCAGATGGTGAGCTGTTGCG-3'; CXCL9 forward, 5'-TTGAGGCTGCAAGGACCC-3' and reverse, 5'-TTTCCCTTCTGGTGTGGTGT-3'; CXCL10 forward, 5'-TGCTGGTCAAGGCTGCTG-3' and reverse, 5'-GGGCCTTGTCAGGCTGATG-3'; TNFSF13B (BLys) forward, 5'-GCAGACAGTGAAACACCA-3' and reverse, 5'-GATGTCCCATGGAAGCTG-3'. The following thermocycling conditions were used: Initial denaturation at 95°C for 30 sec followed by 35 cycles at 95°C for 5 sec and 60°C for 30 sec. Relative mRNA expression levels were calculated using the 2^ΔΔCq method (45).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc.) or R v3.6.3 package (https://www.r-project.org/). The Mann-Whitney U test (Wilcoxon rank sum test) was used to compare two groups which did not meet normal distribution, including Figs. 1B and C, 2B, 4B and 5F. The Kruskal-Wallis test and
a Dwass-Steel-Critchlow-Fligner post hoc test was used to compare multiple groups, which did not meet normal distribution, including Figs. 5A and D, 6A and B, 7A and F and S1. Fisher's exact test was used to analyze the clinicopathological parameters of patients, including TNM stages. The Pearson's $\chi^2$ test was used to analyze differential PSME2 expression associated with the sex of the patients.

Results

PSME2 expression is upregulated in the KIRC dataset from TCGA. To determine the association between PSME2 mRNA expression and KIRC tissues, a transcriptional profile of PSME2, and other PSME genes, was analyzed using TCGA database. The data showed that, compared with that in normal tissues, PSME2 mRNA expression was upregulated in most tumor samples. Furthermore, among the PSME subtypes, PSME1 had a high level of expression. By contrast, the mRNA expression level of PSME4 was low and there was no difference in the expression level of PSME3 between the tumor and normal tissues (Fig. 1A). As shown in Fig. 1B, the median mRNA expression levels of all the subtypes of PSME4 were significantly different between the tumor and normal tissues samples, except for PSME4. PSME1 and PSME2 were upregulated in the tumor tissues, whereas PSME3 was significantly downregulated. Paired tissue analysis also revealed that the expression levels of PSME2 were significantly upregulated (P<0.001) in the tumor samples compared with that in the adjacent normal samples (Fig. 1C). ROC curve analysis was used to investigate the validity of the mRNA expression level in the four PSME genes in KIRC and normal tissues. As shown in Fig. 1D, the AUCs of the four genes were all >60%. The gene with the highest AUC was PSME2 (92.6%). Therefore, the mRNA expression level of PSME2 showed the best specificity and sensitivity to correctly distinguish tumor and adjacent normal kidney samples. Thus, PSME2 may play a critical role in kidney carcinoma.

Upregulated expression of PSME2 in cancer and the prognostic value of PSME2 in patients with ccRCC. To identify the function of PSME2 in different types of tumor, the expression levels between tumor and normal tissues were compared...
using the visualization tools in the Oncomine database. As illustrated in Fig. 2A, the Oncomine database contained 449 unique analyses for PSME2. It was found that PSME2 was upregulated in several types of cancers, including the brain and central nervous system cancer, breast cancer, lymphoma, and pancreatic cancer. A total of three studies revealed a significant increase in mRNA expression levels of PSME2 in kidney cancer samples compared with that in normal samples (46-48).
Accessing TCGA dataset, the PSME2 mRNA expression levels in various types of tumor tissues and adjacent normal tissues were compared (Fig. 2B). It was found that PSME2 was significantly upregulated in bladder urothelial carcinoma, breast invasive carcinoma, cholangiocarcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, colon adenocarcinoma, esophageal carcinoma, glioblastoma multiforme, head and neck squamous cell carcinoma, KIRC, lung adenocarcinoma, liver hepatocellular carcinoma, lung squamous cell carcinoma, prostate adenocarcinoma, rectum adenocarcinoma, stomach adenocarcinoma, thyroid carcinoma and uterine corpus endometrial carcinoma, while it had lower expression levels in pheochromocytoma and paraganglioma compared with that in adjacent normal tissues. In addition, Kaplan-Meier plotter analysis for OS (Fig. 2C), DSS (Fig. 2D) and PFS (Fig. 2E) time revealed an association with PSME2 mRNA expression; therefore, PSME2 was significantly associated with patient prognosis. It was found that patients who had high mRNA expression level of PSME2, had a low survival rate. These findings indicated that PSME2 could be a tumor and prognosis-related marker.

Functional enrichment analysis of PSME2 mRNA expression level in ccRCC. To investigate the molecular mechanisms involved in ccRCC, WGCNA was performed and 194 differentially expressed probes among 12 tumor samples were divided
into five-module colors of the top 25% genes ranked using cluster dendrogram trees, including the colors brown, blue, turquoise, yellow, and grey. The grey module was specified as the gene set that could not be assigned to any module and had no reference meaning (Fig. 3A). The association between module eigengenes and clinicopathological status (normal and abnormal cases) was assessed using a Student’s t-test. The results showed a significant difference in the expression level of module eigengenes between the two groups. **P<0.01. PSME2, proteasome activator complex subunit 2; si, small inhibiting; NC, negative control; GFP, green fluorescent protein.
Figure 6. Effects of PSME2-knockdown on autophagy and tumor cytokine expression in the CAKI-1 and 786-O cell lines. (A) Autophagy-associated proteins were detected using western blot analysis and the results were quantified using densitometry. The data are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01. (B) The mRNA expression level of IL-6, TNF-α, CXCL9, CXCL10 and BLys were determined in the CAKI-1 and 786-O cell lines using reverse transcription-quantitative PCR. *P<0.05, **P<0.01, compared to siNC control. (C) Cell invasion of CAKI-1 and 786-O cell lines transfected with siPSME2 or siNC were analyzed following treatment with Baf-A1. Scale bar, 200 µm. (D) Transmission electron microscopy of autophagosomes in renal carcinoma cells transfected with siRNAs. Left scale bar, 5 µm; right scale bar, 1 µm. PSME2, proteasome activator complex subunit 2; si, small inhibiting; NC, negative control; Baf-A1, bafilomycin A1; SQSTM1, sequestosome 1; BNIP3, BCL2 interacting protein; CXCL, C-X-C motif chemokine ligand; BLys, B lymphocyte stimulator.
CXCL10, and TNFSF9. In addition, the core regulators in the TNF signaling pathway included the CCL5, CXCL10, MMP9, VCAM1 and MMP14 genes. For patients with ccRCC and high expression levels of PSME2, the NF-κB signaling pathway and cytokine-cytokine receptor interaction were enriched in the two gene sets. CXCL9, CCL19, CD27, CSF1R, TNFSF13B and CXCRC4, and CD14, CCL19, TNFSF13B and BCL2A1 were core regulators in the NF-κB signaling pathway and cytokine-cytokine receptor interaction, respectively. Whereas genes in the PSME2 low expression group were enriched in autophagy-animal. BNIP3, GABARAP, ATG10 and MAPK3 genes were found to be hub regulators (Fig. 3F).

Expression level of PSME2 in clinical samples and tumor cells. The bioinformatics results showed that PSME2 expression level was frequently upregulated in KIRC. To verify the results from bioinformatics analysis, the expression patterns of PSME2 were analyzed using IHC and its association with clinicopathological features in patients with kidney cancer was also investigated. Fig. 4A shows representative PSME2 staining images from the TMA. There was strong expression of PSME2 in the cytoplasm of the renal tumor tissues, but a weak expression level in adjacent normal tissues from patients with ccRCC. In addition, univariate analysis showed that T stage (P<0.001) and M stages (P<0.001) were significantly associated with PSME2 expression in the samples from patients with ccRCC. The details of the patient characteristics are listed in Table I. Western blot analysis also revealed that PSME2 protein expression level was more notable in tumor tissues compared with that in matched normal tissues (Fig. 4B). Then, the normal human kidney cell line (HK-2) and the renal carcinoma cell lines (786-O, CAKI-1, ACHN and A498) were used to determine the expression levels of PSME2. As shown in Fig. 4C, PSME2 was increased in the renal tumor cell lines, which was consistent with bioinformatics and IHC results.

Knockdown of PSME2 inhibits the invasive ability of the CAKI-1 and 786-O cell lines. To investigate the effect of PSME2 on cell proliferation and invasion, the 786-O and CAKI-1 cell lines were transfected with siPSME2 and siNC. After transfection for 48 h, the cells were collected and the mRNA expression level of PSME2 was detected using RT-qPCR. As presented in Fig. 5A, after transfection with the 3 siRNAs targeting PSME2, the expression levels of the PSME2 gene in the cells transfected with siPSME2#1 and siPSME2#2 notably decreased compared with that in the control siRNA group (P<0.01). This confirmed that the siPSME2#1 and siPSME2#2 siRNAs were effective. Therefore, siPSME2#1 and siPSME2#2 were selected for further experimentation.

The viability of the 786-O and CAKI-1 cell lines, detected
using a CCK-8 assay, was not significantly different between the siPSME2#1 and siPSME2#2, and siNC groups (Fig. 5B). These data indicated that decreasing the expression level of PSME2 could not inhibit cell proliferation. The role of PSME2 in tumor cell invasion was detected using a Transwell invasion assay. Knockdown of PSME2 in the CAKI-1 and 786-O cell lines reduced invasion compared with that in the cells transfected with siNC (Fig. 5C). Quantitative analysis of the positive stained areas in the Transwell membrane showed that ~35 and 20% of the siPSME2#1-transfected cells were invasive compared with those in the siNC-transfected CAKI-1 and 786-O cells, respectively. Furthermore, 10 and 8% of the cells in the siPSME2#2 group were invasive in the CAKI-1 and 786-O cell lines (P<0.01; Fig. 5D), which suggested that interference of PSME2 reduced the invasive abilities of the cancer cell lines.
Inhibition of PSME2 promotes autophagy and affects the expression level of tumor cytokines in the CAKI-1 and 786-O cell lines. To gain insight into the role of PSME2 in autophagy, PSME2 expression was knocked down using siRNAs, then autophagosomes, which were stained with a specific GFP-LC3 in the 786-O and CAKI-1 cell lines, were analyzed (Fig. 5E and F). The number of autophagosomes per cell was increased following siPSME2#2 transfection, suggesting that knockdown of PSME2 induced autophagy in the CAKI-1 and 786-O cell lines. To determine the status of autophagy in the CAKI-1 and 786-O cell lines following knockdown of PSME2, the protein expression level of the autophagy markers, microtubule-associated protein 1 light chain 3 (LC3), which exists in two forms and transforms from the free form of LC3-I (18 kDa), to the smaller (16 kDa) proteolytic form, LC3-II during autophagy, BNIP3, and SQSTM1 was determined. From western blot analysis (Fig. 6A), LC3-I to LC3-II conversion was elevated and BNIP3 protein expression level was markedly increased in the CAKI-1 cell line. However, SQSTM1 was markedly decreased when the CAKI-1 cell line was transfected with siPSME2#1 or siPSME2#2. Similar results were found in the 786-O cell line. Subsequently, the mRNA expression level of tumor factors associated with immune cells was analyzed using RT-qPCR. IL-6 and TNF-α mRNA expression levels were notably higher in the 786-O and CAKI-1 cell lines transfected with siPSME2 compared with that in the siNC groups. On the contrary, CXCL9, CXCL10 and BLys mRNA expression levels were markedly decreased following transfection with siPSME2 (Fig. 6B). The cells transfected with siPSME2 were then treated with bafilomycin A1 (Baf-A1; an inhibitor of the autophagy) to inhibit autophagy activation. The results of the Transwell assay revealed that invasiveness was increased (Fig. 6C). TEM was used to analyze the autophagosomes, to visualize the effect of siPSME2 on autophagy in the cancer cells. Autophagosomes were characterized by the vacuole-like structure of the bilayer-containing organelles in the cytoplasm. As shown in Fig. 6D, there were a few autophagosomes found in the control cells; however, they were abundant within the cytoplasm of the PSME2-knockout cells. These results indicated that PSME2 induced autophagy.

Effect of overexpression or knockdown of BNIP3 on autophagy and the expression level of tumor cytokines. To investigate the role of BNIP3 in the CAKI-1 cell line and the regulation of autophagy and tumor cytokines, a plasmid vector that overexpressed BNIP3 was constructed and its transfection efficiency was analyzed (Fig. S1). The protein expression levels of BNIP3, PSME2, LC3-I/II and SQSTM1, at 12, 24 and 48 h after transfection and the mRNA expression levels for all the tumor cytokines following the overexpression of BNIP3 (Fig. 7B). To visualize autophagy, LC3 was detected following transfection with GFP-tagged proteins. Overexpression of BNIP3 induced a marked increase in the number of structures labeled by the autophagy marker, as shown in Fig. 7C. Similarly, overexpression of BNIP3 led to an increase in the number of autophagosomes in a time-dependent manner (Fig. 7D). Taken together, the data suggested that
BNIP3 affected autophagy, but had no effect on the mRNA expression level of the tumor cytokines. The Transwell invasion experiments confirmed that knocking down BNIP3 promoted cell invasion, while low PSME2 expression inhibited cell invasion. With inhibition of these two proteins simultaneously, cell invasion was lower compared with that in the control group (Fig. 7E). Western blot analysis confirmed that BNIP3 knockdown suppressed autophagy-related protein expression level and inhibition of PSME2 expression promoted the expression of autophagy-related proteins. Nevertheless, suppressing both proteins, the autophagy inhibited by BNIP3 was restored, as shown in Fig. 7F.

Discussion

Renal cancer is one of the ten most challenging cancer types to diagnose and treat (49). Nearly 90% of kidney tumors are RCC and 80% of cases are ccRCC, making it the most dominant pathological subtype of RCC (50,51). Surgery is still the most effective treatment for local ccRCC; however, 30-35% of patients undergoing surgery will exhibit distant metastasis (50). The prognosis of ccRCC remains unsatisfactory, with a 5-year survival rate of 23% for advanced ccRCC, particularly for locally advanced and metastatic ccRCC (52). Therefore, it is important to investigate a biomarker, which is effective for the treatment and prognosis of ccRCC.

In previous studies, it was found that the role of PSME in tumors has been poorly reported, particularly in ccRCC. The present study described the discovery of aberrant PSME2 mRNA expression in human ccRCC tissues. PSME2 was upregulated in KIRC tissues using TCGA and Oncomine databases, suggesting that overexpression of PSME2 may promote ccRCC. Analysis of DSS, OS and PFS times demonstrated that high PSME2 expression level was predictive of poor perceived prognosis in patients with ccRCC. WGCNA was also used to determine co-expression networks of groups of genes from large expression data and four distinct co-expression modules were identified. The brown module was positively associated with the tumor tissue among the four modules. Furthermore, signal enrichment analysis of the differential genes in the brown module was used to identify GO terms and KEGG and pathways. The GO analysis results revealed the differentially expressed genes were enriched with ‘anion transmembrane transporter activity’, ‘extracellular matrix structural constituent’, ‘apical part of the cell’, ‘collagen containing extracellular matrix’, ‘response to nutrient levels’, ‘extracellular structure organization’, and ‘macromautophagy’. These pathways were associated with tumorigenesis and development. The KEGG pathway analysis showed that these differential genes were associated with ‘autophagy’, ‘complement and coagulation cascades’, ‘rheumatoid arthritis’, ‘protein digestion’, ‘viral protein interaction’, ‘Staphylococcus aureus infection’, ‘cytokine and cytokine receptor’, ‘IL‑17 signaling pathway’, and ‘pertussis’. GSEA showed that PSME2 was associated with the TNF signaling pathway, cytokine-cytokine receptor interaction, autophagy-animal and the NF‑κB signaling pathway in ccRCC. Using a comprehensive analysis of the association between WGCNA and GSEA, the findings indicated that the expression levels of the PSME2 in ccRCC may be involved in cytokine and cytokine receptor interaction and autophagy in the tumor tissue.

Next, the results from bioinformatics analysis were verified. The PSME2 protein expression level was highly expressed in ccRCC tissue, as confirmed using IHC staining and western blot analysis. A previous study reported that PSME2 was a biomarker of tumor invasion and metastasis (30). The cellular experiments then demonstrated that knockdown of PSME2 reduced the invasion of the ccRCC cell lines, but had no effect on cell proliferation. Subsequently, the molecular mechanisms associated with PSME2-induced invasiveness and metastasis of ccRCC were investigated, combined with bioinformatics analysis. Autophagy and tumor cytokine expression levels were investigated in the renal cancer cell lines transfected with or without PSME2-specific siRNA. LC3 is vital for the dynamic process of autophagosome formation. The characteristic signature of autophagic membranes is the conversion of LC3‑I into LC3‑II (53,54). In addition, detecting LC3 using immunofluorescence has become a widely accepted method for identifying autophagy (55). Knockdown of PSME2 resulted in LC3‑I conversion to LC3‑II, which is located on pre-autophagosomes and autophagosomes, making it an autophagy marker (56). Inhibiting PSME2 can promote the formation of autophagosomes. Similarly, more autophagosomes, with GFP‑LC3 fluorescence, were found in the siPSME2 group compared with that in the control group.

BNIP3 is a transmembrane protein, primarily located in the outer membrane of mitochondria. It competes with beclin‑1 to bind to BCL2, releasing beclin‑1 and inducing autophagy (57,58). It was found that the knockdown of PSME2 increased BNIP3 protein expression levels in the 786‑O and CAKI‑1 cell lines. This indicated that inhibiting PSME2 promoted autophagy. SQSTM1 is a scaffold protein in autophagosomes (59) and a stress-inducible protein (60), with multiple domains that mediate its communications with different binding molecules, including a TNF- associated receptor-6 binding domain (61), a Phox1 and Bem1p domain (62), a ZZ-type zinc finger domain, a Keap1-interacting domain, an LC3-interacting domain, and an ubiquitin-associated domain (60,63,64). SQSTM1 is a principle selective autophagy receptor and an important protein in the autophagic clearance of polyubiquitinated proteins (65,66). SQSTM1 binds to the ubiquitinated protein in the autophagosome and fuses with the lysosome to form the autophagosome to be cleared. Likewise, decreased expression of SQSTM1 indicated inhibition of PSME2-induced autophagy. Taken together, we hypothesized that renal cancer cells enhance invasion and inhibit autophagy by overexpressing PSME2.

IL‑6 is a tumor cytokine associated with mortality. High mRNA expression levels of IL‑6 were associated with decreased survival time in patients with ccRCC (67,68). TNF-α is a core adjustor of a complex cytokine network, which not only mediates the pro-inflammatory response, but also regulates the interaction between cells, cell differentiation and cell death (69). A high amount of evidence has indicated that TNF-α has tumor promoting activity (70-73). CXCL9 and CXCL10 are IPN-inducible CXCR3 ligands, and key regulators in recruiting T cells to the tumor microenvironment. It has been reported that patients with ccRCC and a high expression level of CXCL9 and CXCL10 have poor survival times, and are more likely to have early recurrence (74,75).
BLys is a member of the TNF superfamily of ligands and is constitutively expressed on the cell membrane of macrophages, monocytes, activated T cells, dendritic cells, neutrophils, and antigen-presenting cells (76). High BLys protein expression in malignant tumors, including B-cell non-multiple myeloma, Hodgkin’s lymphoma, Hodgkin’s lymphoma and chronic lympho-cytic leukemia, has been reported (77,78). It is noteworthy that IL6 and TNF-α expression levels were increased, but CXCL9/10 and BLys were significantly decreased in ccRCC cell lines transfected with PSME2 siRNA compared with that in the NC siRNA group. However, when BNIP3 was overexpressed, the mRNA expression levels of the five cytokines did not change. It was found that reduced PSME2 expression augments the mRNA expression level of inflammatory factors (IL-6 and TNF-α) and attenuates CXCL9/10 and BLys associated with the tumor microenvironment.

In summary, the findings from the present study demonstrated that PSME2 was upregulated in ccRCC tissues compared with that in normal tissues. This overexpression was not associated with cell proliferation, while it may be associated with cell invasion, autophagy and the tumor microenvironment. The expression of BNIP3 was found via the use of siPSME2 and the effect of BNIP3 overexpression on PSME2 was also found. In addition, the preliminary function of PSME2 in ccRCC was identified using rescue and knockdown experiments. To the best of our knowledge, the present study is the first report the potential significance and function of PSME2 in ccRCC. Future investigations into the molecular mechanism of ccRCC should concentrate on PSME2 and the tumor microenvironment of ccRCC. A limitation to the current study was that only an association between PSME2 and ccRCC was found, additional experiments are required to validate the results. The results provide further information on the effect of PSME2 and the aggressiveness of tumor cells via cytokine and immune cells in ccRCC. In addition, PSME2 could be considered as a biomarker and therapeutic target for ccRCC.

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Availability of data and materials
The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Authors’ contributions
XW, FW, GH, XL designed the research and wrote the manuscript. XW, YD, JC, YZ preformed the bioinformatics analysis and cell experiments. XW and GH analyzed the data and confirmed the authenticity of all the raw data generated during the study. XW, FW, GH and XL contributed to the critical reading and correction of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Ethics committee approval was obtained from the Institutional Ethics Committee of West China Hospital of Sichuan University (approval no. 2021211A) to the commencement of the study laid down in the 1964 Declaration of Helsinki. Written informed consent was provided by all the participants.

Patient consent for publication
Not applicable.

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

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