Postprint: Study on the Association of Cathepsin S with Tumor Progression and Prognosis

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Abstract

Background: Cathepsin S (CTSS) is a lysosomal cysteine protease with upregulated expression levels in various tumor tissues, but the relationship between CTSS levels and tumor progression and prognosis remains unclear.

Objective: To investigate the expression differences of CTSS in pan-cancer tissues and clarify the correlation between CTSS levels and tumor progression and prognosis.

Methods: RNA-Seq gene expression profiles of 33 cancer tissues and related clinical data were downloaded from the TCGA database, and CTSS gene expression data in human normal tissues were collected from the HPA database. R packages were utilized to visually analyze CTSS expression differences and their relationship with overall survival (OS) of cancer patients, and to explore the correlation between CTSS and tumor mutational burden (TMB), microsatellite instability (MSI), and immune checkpoints; Western blot was employed to detect CTSS expression differences in normal glial cell line (HA1800) and three glioma cell lines (C6, U87, U251); CCK-8 assay was used to verify the effect of CTSS expression on cell proliferation, and one-way ANOVA was applied to analyze inter-group differences.

Results: CTSS levels were lower in colon cancer, lung adenocarcinoma, non-small cell lung cancer, pancreatic cancer, prostate cancer, and rectal cancer tissues compared to normal tissues, while CTSS expression levels were higher in ovarian cancer, glioblastoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, gastric adenocarcinoma, thyroid cancer, and endometrial cancer than in normal tissues, with all differences being statistically significant ($P<0.05$). Survival analysis results demonstrated that in bladder transitional cell carcinoma, lower-grade glioma, ovarian serous cystadenocarcinoma, sarcomatoid lung carcinoma, melanoma, and uveal melanoma, the OS comparison between patients with high and low CTSS expression showed statistically significant differences ($P<0.05$). Correlation analysis results revealed that CTSS

levels were positively correlated with TMB in lower-grade glioma, esophageal cancer, and breast cancer (P<0.05); CTSS levels were negatively correlated with TMB in non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, and head and neck cancer (P<0.05); CTSS levels were negatively correlated with MSI in testicular cancer, melanoma, pheochromocytoma, pancreatic cancer, ovarian cancer, non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, lower-grade glioma, papillary renal cell carcinoma, head and neck cancer, and diffuse large B-cell lymphoma (P<0.05); CTSS levels were positively correlated with TNFRSF9, CD86, HAVCR2, and CD200R1 in 33 tumor tissues (P<0.05). Western blot results showed that CTSS levels in HA1800 cells were lower than those in the three malignant glioma cell lines, with statistically significant differences (P<0.05). CCK-8 assay results indicated that after 48 and 72 hours of culture, the proliferation capacity of cells in the shRNA interference group was lower than that in the control and empty vector groups for HA1800, C6, U87, and U251 cells, with all results being statistically significant (P<0.05).

Conclusion: CTSS is associated with multiple tumor immune checkpoints, MSI, and TMB. High CTSS expression can promote tumor cell proliferation and reduce patient survival, serving as a biomarker for poor prognosis in various cancers.

Full Text

Correlation Analysis between Cathepsin S and Tumor Progression and Prognosis

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Abstract

Background: Cathepsin S (CTSS) is a lysosomal cysteine protease that exhibits upregulated expression in various tumor tissues. However, the relationship between CTSS levels and tumor progression and prognosis remains unclear.

Objective: To investigate the differential expression of CTSS across pan-cancer tissues and to clarify the correlation between CTSS levels and tumor progression and prognosis.

Methods: RNA-Seq gene expression profiles and clinical data for 33 cancer types were downloaded from The Cancer Genome Atlas (TCGA) database. CTSS gene expression data in normal human tissues were collected from the

Human Protein Atlas (HPA) database. R packages were used to visualize and analyze CTSS expression differences and their relationship with overall survival (OS) in cancer patients. The correlations between CTSS and tumor mutational burden (TMB), microsatellite instability (MSI), and immune checkpoints were investigated. Western blot was used to detect CTSS expression differences between normal glial cell line (HA1800) and three glioma cell lines (C6, U87, U251). The effect of CTSS expression on cell proliferation was verified using CCK-8 assay. One-way ANOVA was used to analyze intergroup differences.

Results: CTSS levels were significantly lower in colon cancer, lung adenocarcinoma, non-small cell lung cancer, pancreatic cancer, prostate cancer, and rectal cancer tissues compared to normal tissues, while CTSS expression was significantly higher in ovarian cancer, glioblastoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, gastric adenocarcinoma, thyroid cancer, and endometrial cancer ($P<0.05$). Survival analysis revealed statistically significant differences in OS between high and low CTSS expression groups in bladder transitional cell carcinoma, low-grade glioma, ovarian serous cystadenocarcinoma, sarcomatoid lung cancer, melanoma, and uveal melanoma ($P<0.05$). Correlation analysis showed that CTSS levels were positively correlated with TMB in low-grade glioma, esophageal cancer, and breast cancer ($P<0.05$), but negatively correlated with TMB in non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, and head and neck cancer ($P<0.05$). In testicular cancer, melanoma, pheochromocytoma, pancreatic cancer, ovarian cancer, non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, low-grade glioma, papillary renal cell carcinoma, head and neck cancer, and diffuse large B-cell lymphoma, CTSS levels were negatively correlated with MSI ($P<0.05$). Across 33 tumor types, CTSS levels were positively correlated with TNFRSF9, CD86, HAVCR2, and CD200R1 ($P<0.05$). Western blot results showed that CTSS levels in HA1800 cells were significantly lower than in the three malignant glioma cell lines ($P<0.05$). CCK-8 assays demonstrated that the proliferation ability of cells in the shRNA interference group was significantly lower than in the control and empty vector groups after 48 and 72 hours of culture in HA1800, C6, U87, and U251 cells ($P<0.05$).

Conclusion: CTSS is associated with various tumor immune checkpoints, MSI, and TMB. High CTSS expression can promote tumor cell proliferation and reduce patient survival, serving as a biomarker for poor prognosis in multiple cancers.

Keywords: Carcinoma; Cathepsin S; Biomarker; Prognosis; Immune infiltration

Introduction

With advances in medicine, tumor treatment has expanded beyond surgery and traditional chemoradiotherapy to include novel therapeutic approaches such as

gene-targeted therapy, monoclonal antibody/antibody-drug conjugate therapy, cellular immunotherapy, and immune checkpoint inhibitors. However, the treatment of cancer, particularly in relapsed and refractory patients, remains a critical challenge [1]. Studies have shown that oncogenes can promote tumor development, while tumor suppressor genes play important roles in anti-tumor therapy [2-4]. Regulating relevant oncogenes and tumor suppressor genes for anti-tumor treatment represents a major focus in both research and clinical practice [5].

The development and application of immune checkpoint inhibitors have improved treatment response rates and reduced tumor recurrence in patients with certain solid tumors such as melanoma, pancreatic cancer, and lung cancer, as well as hematological malignancies like non-Hodgkin lymphoma [6-7]. However, many patients still exhibit low sensitivity to tumor immunotherapy, necessitating the exploration of novel and reliable immune checkpoints to achieve better anti-tumor efficacy [8].

Cathepsin S (CTSS) is a group of intracellular peptide hydrolases primarily located in lysosomes and belongs to the cysteine protease family. Research has confirmed that CTSS expression is upregulated in glioblastoma, gastric cancer, and non-Hodgkin lymphoma tissues [9-11]. Further studies have revealed that CTSS is a key factor in the proliferation, invasion, and metastasis of glioblastoma, gastric cancer, and non-Hodgkin lymphoma tissues, and that CTSS overexpression is associated with tumor angiogenesis [12]. These results indicate that CTSS levels are closely related to poor prognosis in cancer patients and may serve as a potential therapeutic target. However, the correlation between CTSS expression and pan-cancer progression and prognosis remains to be elucidated. This study investigated CTSS expression differences between pan-cancer and normal tissues, analyzed correlations between CTSS and tumor immune infiltration, tumor mutational burden (TMB), microsatellite instability (MSI), and immune checkpoints, and evaluated its relationship with tumor progression and prognosis to provide a reference basis for clinical treatment and prognostic assessment of pan-cancer.

Methods

Data Resources and Processing

RNA sequencing (RNA-Seq) data, phenotype data, and survival data for 33 tumor types (44,736 cases) were collected from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/ccg/research/genome-sequencing/tcga), including adrenocortical carcinoma, bladder transitional cell carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma, cholangiocarcinoma, colon cancer, diffuse large B-cell lymphoma, esophageal cancer, glioblastoma, head and neck squamous cell carcinoma, kidney chromophobe carcinoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, acute myeloid leukemia, low-grade glioma, hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, mesothelioma, ovarian serous cystadenocarcinoma, pan-

creatic cancer, pheochromocytoma and paraganglioma, prostate cancer, rectal adenocarcinoma, sarcomatoid lung cancer, cutaneous melanoma, gastric cancer, testicular cancer, thyroid cancer, thymic cancer, endometrial cancer, uterine sarcoma, and uveal melanoma [13]. The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/) provides data on human protein expression, function, and cellular/tissue/organ localization [14]. CTSS gene expression data in normal human tissues (6,396 cases) were collected from the HPA database.

Survival Analysis

Using the "survival" and "survminer" packages in R version 4.1.1, survival curves were plotted to analyze the relationship between CTSS levels and overall survival (OS) in cancer patients. Patients were divided into high and low expression groups based on CTSS expression levels, and OS was compared between these groups.

Correlation Analysis of CTSS Levels with TMB and MSI

Tumor mutational burden (TMB) is a quantitative assessment of somatic mutations within the tumor genome and can serve as a biomarker for susceptibility to immune checkpoint inhibitor therapy in certain cancers [15]. High microsatellite instability (MSI-H) refers to the gain or loss of nucleotides in repetitive DNA due to genomic hypermutation, which can be used as a diagnostic phenotype to predict response to immune checkpoint inhibitor therapy [16]. The ESTIMATE algorithm was used to evaluate tumor immune scores, TMB scores were calculated as the proportion of mutated genes, and MSI scores were calculated as the number of unstable microsatellite sequences. The "fmsb" package in R version 4.1.1 was used for correlation analysis between TMB/MSI scores and CTSS levels, with results visualized using radar maps.

Correlation between CTSS Expression and Immune Checkpoints

CTSS levels are associated with the expression of immune checkpoint molecules such as CD44, CD86, and CD70 [17]. Using the "reshape2" and "RColorBrewer" packages in R version 4.1.1, heatmaps were generated to analyze the correlation between CTSS levels and immune checkpoints including TNFRSF9, CD44, CD86, CD274, TIGIT, TNFSF, CD40, VSIR, CD27, BTNL2, HHLA2, PDCD1LG2, IDO, VTCN1, TMIGD2, ICOSLG, CD160, LGALS9, PDCD1, CD80, KIR3DL1, CD276, ADORA2A, HAVCR2, CD200R1, CD28, CD48, CTLA4, CD40LG, ICOS, LAG3, CD244, TNFSF4, LAIR1, NRP1, TNFRSF14, CD200, and BTLA.

Cell Culture, Transfection, and Identification

Normal glial cell line HA1800 (Suzhou Haixing Biological Company) and glioma cell line C6 (Suzhou Haixing Biological Company) were cultured in F12K medium containing 15% horse serum, 2.5% fetal bovine serum (FBS),

and 1% penicillin-streptomycin. Glioma cell lines U87 and U251 (Suzhou Haixing Biological Company) were cultured in DMEM containing 10% FBS at 37°C with 5% CO_2 and saturated humidity. The experiment was divided into shRNA interference group, empty vector group, and control group. Cells in logarithmic growth phase were used; for the shRNA interference group, 1.5$×10^{5}$ wild-type cells were seeded in 6-well plates 12 hours before transfection. After 24 hours of culture, Lipofectamine™ 3000 transfection reagent (Wuhan Servicebio Technology Co., Ltd.) was used to transfect shRNA plasmid (pLVX-CTSSshRNA-P) at 2 g/well into wild-type cells. The empty vector group was transfected with pLVX-AcGFP1-N empty plasmid at 2 g/well using Lipofectamine™ 3000. The control group was cultured in complete medium for 24 hours. After 24 hours of transfection, cells were collected from each group for Western blot detection of intracellular CTSS protein expression and CCK-8 assay for relative proliferation rates.

Western Blot Detection

Cells in each group were fully lysed using RIPA lysis buffer (Wuhan Servicebio Technology Co., Ltd.), centrifuged, and supernatants were collected. Protein concentrations were determined using BCA protein assay kit (Wuhan Servicebio Technology Co., Ltd.). Electrophoresis loading buffer was prepared for electrophoresis and membrane transfer. After blocking with 5% skim milk for 2 hours, primary antibodies for CTSS and GAPDH (Wuhan Servicebio Technology Co., Ltd.) were added at 1:1000 dilution and incubated overnight at 4°C. The next day, after washing with TBST buffer, secondary antibody (goat anti-mouse IgG, Wuhan Servicebio Technology Co., Ltd.) was added at 1:2000 dilution and incubated for 2 hours. Developer solution was prepared for imaging, and quantitative analysis was performed using Image J 1.8.0 [18].

CCK-8 Assay

Cells in logarithmic growth phase were seeded in 96-well plates at a density of $5\$\times10\hat{}\{4\}/ml(100\mu l/well)withthreereplicatewellspergroup.After24, 48, and72hoursofcultureat37°Cwith5\_$ CCK-8 reagent (Wuhan Servicebio Technology Co., Ltd.) was added at 10 l/well and incubated for an additional 2 hours. Absorbance at 450 nm was measured to calculate relative cell proliferation rates.

Statistical Methods

Statistical analysis of data collected from TCGA and HPA databases was performed using R version 4.1.1. Differences in CTSS expression and proliferation among different cell lines were analyzed using GraphPad Prism 9.3.1. Normally distributed measurement data were expressed as ($\bar{x}\pm s$), and intergroup comparisons were performed using one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Clinical Data and Preprocessing

RNA-seq data, phenotype data, and survival data for 33 tumor types (44,736 cases) were downloaded from the TCGA database. Probes were converted to gene symbols according to annotation documents. After measuring median probe expression values, duplicate probes were removed. Samples lacking survival data and gene expression data were excluded, resulting in 28,942 samples for analysis.

Comparison of CTSS Levels Between Normal and Cancer Tissues

Analysis of CTSS expression in normal and tumor tissues revealed that CTSS levels were significantly lower in colon cancer, lung adenocarcinoma, non-small cell lung cancer, pancreatic cancer, prostate cancer, and rectal cancer tissues compared to normal tissues ($P<0.05$). In contrast, CTSS expression was significantly higher in ovarian cancer, glioblastoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, gastric adenocarcinoma, thyroid cancer, and endometrial cancer ($P<0.05$) [Figure 1: see original paper].

Survival Analysis of Patients with Different CTSS Levels

Survival analysis showed statistically significant differences in OS between high and low CTSS expression groups in bladder transitional cell carcinoma, low-grade glioma, ovarian serous cystadenocarcinoma, sarcomatoid lung cancer, melanoma, and uveal melanoma ($P<0.05$). No statistically significant differences in OS were observed between high and low expression groups in adrenocortical carcinoma, breast invasive carcinoma, cervical squamous cell carcinoma, cholangiocarcinoma, colon cancer, diffuse large B-cell lymphoma, esophageal cancer, glioblastoma, head and neck squamous cell carcinoma, kidney chromophobe carcinoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, acute myeloid leukemia, hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, mesothelioma, pancreatic cancer, pheochromocytoma and paraganglioma, prostate cancer, rectal adenocarcinoma, gastric cancer, testicular cancer, thyroid cancer, thymic cancer, endometrial cancer, or uterine sarcoma ($P>0.05$) [Figure 2: see original paper].

Correlation Analysis Between CTSS and TMB/MSI

The mismatch repair pathway plays a crucial role in repairing DNA replication errors in both normal and cancer cells. DNA mismatch repair deficiency and secondary microsatellite instability can increase the burden of oncogene mutations [19]. Correlation analysis revealed that CTSS levels were positively correlated with TMB in low-grade glioma, esophageal cancer, and breast cancer ($P<0.05$), but negatively correlated with TMB in non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, and head and neck cancer ($P<0.05$).

In testicular cancer, melanoma, pheochromocytoma, pancreatic cancer, ovarian cancer, non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, low-grade glioma, papillary renal cell carcinoma, head and neck cancer, and diffuse large B-cell lymphoma, CTSS levels were negatively correlated with MSI ($P<0.05$) [Figure 3: see original paper].

Correlation Between CTSS and Immune Checkpoints

Correlation analysis showed that CTSS levels were positively correlated with TNFRSF9, CD86, HAVCR2, and CD200R1 across 33 tumor types ($P<0.05$) [Figure 4: see original paper].

Comparison of CTSS Levels in Different Cell Lines

Western blot results demonstrated significant differences in relative CTSS expression levels among the four cell lines ($P<0.05$). Normal glial cell line HA1800 showed significantly lower CTSS expression levels compared to the three malignant glioma cell lines (C6, U87, and U251) ($P<0.05$) [Figure 5: see original paper], .

CCK-8 assays showed that after 24, 48, and 72 hours of culture, significant differences in cell proliferation rates were observed among control, empty vector, and shRNA interference groups in U87 and U251 cell lines ($P<0.05$), with the shRNA interference group showing lower proliferation than control and empty vector groups ($P<0.05$). After 48 and 72 hours of culture, significant differences were also observed in C6 cell lines ($P<0.05$), with the shRNA interference group showing lower proliferation than control and empty vector groups ($P<0.05$) .

Discussion

Immune checkpoints play important roles in activating or inhibiting T cell function [20-21]. Tumor cells can evade immune system attack through immune checkpoint regulation, while immune checkpoint blockade therapy can enhance host immune system attack on tumor cells by inhibiting programmed death receptor-ligand binding [22-23]. This study evaluated CTSS expression levels across 33 cancer types and analyzed correlations between CTSS and multiple immune checkpoints, providing an important basis for CTSS application in pan-cancer therapy.

We found that CTSS levels were lower in colon cancer, lung adenocarcinoma, non-small cell lung cancer, pancreatic cancer, prostate cancer, and rectal cancer, but higher in ovarian cancer, glioblastoma, clear cell renal cell carcinoma, papillary renal cell carcinoma, gastric adenocarcinoma, thyroid cancer, and endometrial cancer, consistent with previous studies [24]. Survival analysis revealed that high CTSS levels were associated with poorer OS in patients with low-grade glioma, pancreatic cancer, thyroid cancer, and uveal melanoma, indicating that CTSS is an important factor affecting survival in multiple tumor

types [25-26]. Previous studies have shown that CTSS is highly expressed in mucinous carcinoma and thyroid papillary tumor cells, with expression levels positively correlated with tumor development, angiogenesis, invasion, and metastasis [27-29]. Our study found that CTSS was highly expressed in glioblastoma, low-grade glioma, uveal melanoma, and kidney chromophobe carcinoma, correlating with poor tumor prognosis.

The mismatch repair pathway is crucial for repairing DNA replication errors in normal and cancer cells. DNA mismatch repair deficiency and secondary MSI can increase the burden of oncogene mutations [19]. We found that CTSS expression was positively correlated with TMB in low-grade glioma, esophageal cancer, and breast cancer, but negatively correlated with TMB in non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, and head and neck cancer. CTSS expression was negatively correlated with MSI in testicular cancer, melanoma, pheochromocytoma, pancreatic cancer, ovarian cancer, non-small cell lung cancer, lung adenocarcinoma, hepatocellular carcinoma, low-grade glioma, papillary renal cell carcinoma, head and neck cancer, and diffuse large B-cell lymphoma.

Immune checkpoint inhibitors, particularly antibodies against CTLA-4, programmed death protein-1 (PD-1) and its ligand (PD-L1), are widely used in clinical treatment of solid tumors such as melanoma, non-small cell lung cancer, and non-Hodgkin lymphoma, with PD-1/PD-L1 inhibitor therapy currently covering 17 cancer indications [30-31]. Our study found that across 33 cancer types, CTSS was correlated with most immune checkpoints including CTLA-4, CD200R1, HAVCR2, CD86, and TNFRSF9, particularly showing positive correlations with TNRDF9, CD44, CD86, CD274, and CD28 in high- and low-grade gliomas. Regulating immune checkpoints through CTSS expression may provide a reference basis for tumor development and anti-tumor drug development.

We found that CTSS levels were lower in normal glial cell line HA1800 compared to three malignant glioma cell lines (C6, U87, and U251), indicating low CTSS expression in normal glial cells but high expression in malignant glioma. Using CTSS plasmid to construct shRNA interference cell models, we found that the proliferation ability of malignant glioma cells in the interference group was lower than in control and empty vector groups, demonstrating that CTSS can promote glioma cell proliferation and that interference with CTSS expression reduces cell proliferation ability, indicating a positive correlation between CTSS and glioma cell proliferation.

This study has several limitations. First, the samples were derived from databases without single-center or multi-center clinical samples, and the large number of cancer types limited the depth of analysis for individual cancer types. Second, the basic experimental validation did not investigate CTSS-related signaling pathways, which requires further improvement.

In conclusion, high CTSS expression can promote tumor cell proliferation and reduce patient survival, serving as a biomarker for poor prognosis in multiple

tumors, particularly glioma. CTSS is closely associated with various tumor immune checkpoints, and targeting CTSS for immune checkpoint blockade therapy may represent an important strategy for inhibiting tumor development. This study provides a reference basis for developing novel immune checkpoint inhibitors for tumor immunotherapy.

Author Contributions: Jing Li was responsible for bioinformatics and experimental work, data collection, and manuscript writing. Yu Liu performed data visualization. Zongren Zhao revised and polished the manuscript. Jinyu Zheng was responsible for data analysis. Rutong Yu designed the study, implemented the protocol, and reviewed the manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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Note: Figure translations are in progress. See original paper for figures.