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Cathepsin E expression and activity: Role in the detection and treatment of pancreatic cancer

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ABSTRACT

Cathepsin E (CTSE) is an intracellular, hydrolytic aspartic protease found to be expressed in cells of the immune and gastrointestinal systems, lymphoid tissues, erythrocytes, and cancer cells. The precise functions are not fully understood; however, various studies have investigated its numerous cell-type specific roles. CTSE expression has been shown to be a potential early biomarker for pancreatic ductal adenocarcinoma (PDAC). PDAC patients have low survival rates mostly due to the lack of early detection methods. CTSE-specific activity probes have been developed and tested to assist in tumor imaging and functional studies investigating the role of CTSE expression in PDAC tumors. Furthermore, a CTSE protease-specific, photodynamic therapy pro-drug was developed to explore its potential use to treat tumors that express CTSE. Since CTSE is expressed in pancreatic diseases that are risk factors for PDAC, such as pancreatic cysts and chronic pancreatitis, learning about its function in these disease types could assist in early PDAC detection and in understanding the biology of PDAC progression. Overall, CTSE expression and activity shows potential to detect PDAC and other pancreatic diseases. Further research is needed to fully understand its functions and potential translational applicability.

Introduction

Cathepsin E (CTSE) is an aspartic protease and a member of the peptidase A1 family of proteases, along with pepsin A and cathepsin D (CTSD) [1]. Although CTSE was initially found to be expressed in the spleen, it has recently been discovered to be expressed intracellularly in a variety of cell types, including gastrointestinal cells, antigen-presenting cells of the immune system, and several cell types within lymphoid tissues [2–4]. In some immune cell types (dendritic, microglia, and macrophages), CTSE is found to be expressed in the endosomal compartments, while in erythrocytes and gastric cells, CTSE is expressed in the plasma membrane [4–7]. As a protease, the main function of CTSE is to break down proteins through the hydrolysis of peptide bonds at a specific peptide sequence site [8]. The discovery of the functional specificity of CTSE protease activity, along with increased expression in various diseases, has led to increased interest towards studying CTSE. Studies have focused on using CTSE to improve disease diagnostics, develop imaging agents, and enhancement of treatment modalities in several types of cancers, including pancreatic ductal adenocarcinoma (PDAC) [9–14].

PDAC is currently the third leading cause of cancer-related deaths, with a 5-year survival rate of 9% [15]. Part of this poor prognosis is due to the current inability to detect early-stage disease, when it is potentially resectable [16]. These challenges have led to increased efforts toward the discovery and validation of biomarkers for detecting PDAC at an early stage [17,18]. Among them, CTSE has been identified as a promising biomarker for several diseases. It is highly expressed in PDAC tissues [13,19] as well as gastric [20,21], cervical [22], esophageal [23], lung adenocarcinomas [24], squamous cell carcinoma [24], bladder [25,26], and ovarian carcinoma [27]. Moreover, besides expression levels of CTSE, the proteolytic activity of CTSE could be used for the early

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detection and diagnosis of multiple malignancies and has shown promising results in the development of cancer-directed chemotherapeutics [1,10,12,13,28]. Future discoveries of CTSE's function in cancer and the pathways it modulates will enable the development of novel therapeutics and imaging modalities for treating and detecting PDAC. Therefore, here a comprehensive literature review was performed in order to organize and assess the state of CTSE research and how it relates to PDAC. This review discusses current knowledge of the biology and function of CTSE, as well as findings that link CTSE expression and activity to aspects of PDAC and other pancreatic diseases that increase the risk of PDAC development.

Physiological functions of cathepsin E

There are many gaps in knowledge as to the exact physiological functions of CTSE, though there are many hypotheses about the role CTSE expression plays in assorted cell types. In 1989, Sakai et al. examined the distribution of CTSE expression throughout various organs and tissues in rats and postulated possible functions based on its cellular localization [29]. Initially, it was thought that since CTSE was expressed abundantly in foveolar epithelial cells of the stomach and in lymph nodes, that it was then involved in the turnover of rapidly regenerating cells and in the processing of protein antigens for the immune response [29]. Further research showed that CTSE expression in erythrocytes was associated with localization to the plasma membrane and became activated by cellular aging that induced enzyme solubilization [30]. When activated, CTSE began to break down plasma membrane proteins as part of senescence-induced mechanisms or damaged to red blood cells [29,30].

To further understand some of the physiological functions of CTSE expression in the body, Tsukuba et al. studied CTSE-deficient mice which developed atopic dermatitis (AD) [31]. Lack of CTSE expression in these mice reduced the turnover rates of the pro-inflammatory cytokines interleukin-18 and interleukin-1β [31]. Since similar results were not seen in mice reared under pathogen-free conditions, the AD observed was likely due to an increased susceptibility to bacterial infection and the activation of various immune-related pro-inflammatory pathways [31]. In addition, erythrocytes derived from patients or mice with AD showed reduced CTSE expression, suggesting that a defect in the production of CTSE may lead to the development of AD [31]. Moreover, a different group associated CTSE deficiency with adipose tissue modulation since CTSE-deficient mice fed a high-fat diet had an impairment in adipose tissue development that was linked to a reduction in macrophage infiltration [32]. CTSE was later found to have another function in modulating immune cells, as it was the major aspartic protease involved in antigen processing via the major histocompatibility complex class II (MHC-II) pathway in a B cell lymphoblast cell line [33]. Further functional studies that utilized a selective and highly specific CTSE inhibitor isolated from the nematode *Ascaris lumbricoides* demonstrated that CTSE was essential in the processing of ovalbumin (OVA) by B cell lymphoblasts for later presentation to OVA-specific T cells [33]. In 2002, CTSE was found to have a similar role in the MHC-II antigen-presenting pathway in microglia through a similar mechanism [5]. Most of the functions of CTSE at that time described functions related to normal physiology; however, increased expression of CTSE in various cancers was starting to emerge.

Since upregulation of CTSE expression has been associated with multiple cancers, researchers in cancer biology have focused on uncovering novel CTSE functions and their potential for therapeutic targeting. In prostate cancer, CTSE expression was associated with anti-tumor properties: CTSE decreased tumor growth and increased apoptosis by catalyzing the release of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) from the surface of tumor cells [34]. Moreover, combined CTSE and doxorubicin treatments assisted in overcoming chemoresistance of prostate cancer cells *in vitro* [35]. Another group showed that upregulation of CTSE, induced growth arrest of prostate cancer cells by inhibiting angiogenesis, and was associated increased levels of antiangiogenic molecules, such as interleukin-12 (IL-12), monokine induced by g-interferon (MIG), and endostatin [36]. While CTSE has been found to have several functions in prostate cancer that are anti-tumorigenic, other cathepsins (e.g., cathepsin L, cathepsin S, and cathepsin D (CTSD)) are thought to enhance metastasis by breaking down extracellular structures [37,38]. There is also evidence that CTSE might behave differently in other cancers. For example, in pancreatic cancer, the abundance and localization of CTSE expression may have minimal function, either due to a lack of activation or from increased expression of CTSE-related inhibitory factors [39]. Since few studies have focused on understanding the function of CTSE in cancer and have primarily focused on prostate cancer, there is a knowledge gap regarding the function and mechanisms by which multiple cancer cell types upregulate the expression of CTSE. The development of CTSE-specific inhibitors will help elucidate the function of CTSE in different cancers and determine whether CTSE could be a potential therapeutic target for cancer treatment.

Selective inhibition of cathepsin E activity

Finding an inhibitor that specifically and selectively targets CTSE rather than other aspartic proteases is essential when investigating the function of CTSE in the cell and for therapeutic development. Currently, pepstatin is most commonly used when trying to specifically inhibit CTSE. It is an aspartic protease inhibitor that inhibits pepsin in actinomycetes [40]. However, as an unspecific aspartic protease inhibitor, pepstatin inhibits other targets, including CTSD, pepsin, and several other proteases, so it doesn't allow for the specific assessment of CTSE functions [5,41].

Several small molecule inhibitors have been developed that specifically inhibit CTSD and CTSE but cannot differentiate between them [41]. In 1972, a compound was isolated from the parasitic worm *Ascaris lumbricoides* and was found to specifically inhibit CTSE [42]. This compound; however, could not be produced in sufficient quantities for use in functional studies. Recently, several other CTSE inhibitors have been found in cyanobacteria [43,44]. One of these compounds, grassystatin A, found by Kwan et al., has 38-fold selectivity for CTSE compared to CTSD [44]. A method of synthesis has recently been developed to produce grassystatin A, which may be useful to study CTSE function [45]. While examining the function of CTSE in regulating protein turnover of the α2-macroglobulin (a protease inhibitor and transporter), researchers found that the amino acid alanine position of a peptide substrate was essential for CTSE-selective cleavage [46]. This finding has assisted in the development of CTSE-specific peptide sequence probes for detecting and quantifying CTSE activity [14,46,47], and for treatment targets [48,49].

Cathepsin E as a biomarker in pancreatic cancer

One factor leading to the devastating prognosis of PDAC is the lack of effective methods for early detection. PDAC is often not diagnosed until the disease has spread locally or distally, and surgical resection is a possibility only in <20% of all patients [16]. Serum levels of carbohydrate antigen 19–9 (CA19-9) are used for diagnosis of PDAC, but has poor diagnostic accuracy, especially for early stages of disease when it is particularly unreliable. Discovery of a biomarker that will improve the accuracy of PDAC detection may result in earlier detection and greater chances for long term

survival.

CTSE was first found to be expressed in PDAC through immunohistochemical staining (IHC) by Sessa et al., in 1990, detecting CTSE expression in 92% of resected tumors [9]. Further IHC in a small group of subjects showed CTSE to be upregulated in PDAC tissue compared to chronic pancreatitis tissue [19]. Early pancreatic intraepithelial neoplasia (PanIN) lesions showed CTSE staining, suggesting the possible use of CTSE as an early biomarker for this disease [13,19]. CTSE has also been measured via enzyme-linked immunosorbent assay (ELISA) and western blots, using pancreatic fluid obtained via endoscopy; CTSE performed better than CA19-9, CEA, and the expression of *Kras* mutations in differentiating PDAC from chronic pancreatitis [10,19]. While PDAC detection using CTSE expression in pancreatic fluid shows promise in diagnostic testing, an invasive procedure is required to collect the fluid. Moreover, with some of the tools currently available, CTSE expression has not been detected or analyzed in blood or other biofluids of patients with any pancreatic disease [19,23]. One less invasive method to detect CTSE in pancreatic tissues involves using a fluorescent probe selective for the detection of CTSE activity via optical imaging. Using this CTSE-specific fluorescent peptide probe, CTSE activity was detected in PanIN lesions using various preclinical murine models (a human xenograft model and several genetically engineered mouse models of PDAC) [13].

Cathepsin E activity imaging peptide probes

Several different approaches have been used to develop probes that can accurately detect CTSE activity and expression levels both *in vitro* and *in vivo* [13,14,47,50]. In 1999, Weissleder et al. theorized that certain tumors could be detected due to their high protease expression, and experimented with a fluorescent probe activated by protease activity in a xenograft mouse model of breast cancer that was successful in detecting and imaging tumors [51]. The probe itself was activated through enzymatic cleavage of a protease in lysosomes [51]. Multiple molecules of Cy5.5, a near-infrared fluorophore, were attached to a copolymer, which assisted in localizing the probe to the tumor. Initially, the probe's fluorescence was quenched due to the fluorophores being within close proximity of each other. Once the fluorophores were cleaved and separated from each other, a 12-fold increase in fluorescent signal was observed, which could be used to image tumors *in vivo* [51].

Weissleder's imaging strategy utilized a probe activated by tumor-associated proteases, but it was not fully specific for the tumor. In order to gain specificity, researchers needed to develop a probe that was activated by tumor-specific proteases which were more distinctly overexpressed, such as CTSE. Many of the early CTSE probes struggled to differentiate between CTSE and CTSD due to them sharing many structural similarities, including the catalytic mechanism and substrate preference [50]. However, a few CTSE-selective peptide substrates were discovered based on their selective cleavage of molecules such as α2-macroglobulin [52]. Yasuda et al. confirmed CTSE's selectivity for the new substrates using an *in vitro* fluorescent probe model, specifically proving CTSE's ability to preferentially cleave the substrate rather than CTSD [52].

Utilizing CTSE-specific substrates, multiple CTSE-specific probes have been synthesized in order to achieve *in vitro* and *in vivo* detection. In 2010, several potential CTSE probes were synthesized and tested *in vitro* among a variety of cathepsins (most notably, cathepsins D and E) [47]. The peptide sequence Mca-Ala-Gly-Phe-Ser-Leu-Pro-Ala-Lys (Dnp)-DArg-CONH2 probe was found to displayed high selectivity for CTSE [47]. The fluorescent probe comprised of the fluorescent donor 7-methoxycoumarin-4-acetic acid and energy acceptor dinitrophenyl (Dnp) was created specifically for *in vitro* use, as it is too small to be used *in vivo* [47]. However, using Weissleder's approach, a CTSE-selective imaging probe was synthesized and tested *in vivo* using both human xenografts and various genetically-engineered mouse models [13,14]. Utilizing a probe in which multiple CTSE-specific peptide substrates were attached to a polylysine backbone and labeled with Cy5.5 as the fluorescent donor, detection of PDAC tumors in mice was achieved. The addition of the peptide substrates to a backbone created a larger probe molecule, ultimately aiding in tissue diffusion and cell membrane translocation. With further validation, these CTSE activity probes could be translated into clinical use for imaging or a clinical sample bioassay for cancer detection.

Future research should examine the feasibility of combining CTSE probes with other forms of *in vivo* imaging to improve their clinical utility. For example, Li et al. combined CTSE activity probe technology with confocal laser endomicroscopy (CLE) in order to visualize tumors *in vivo* [11]. They used CLE [11] and a CTSE activity probe [13] to study the progression of PDAC tumorigenesis within mouse models, providing the foundation for diagnosing PDAC tumors that is less invasive than biopsy or obtaining pancreatic fluid samples.

Cathepsin E activity as a therapeutic activator

Photodynamic therapy (PDT) is a widely accepted, minimally invasive treatment for both cancerous and precancerous lesions. Using a photosensitizer, light, and oxygen, PDT generates reactive oxygen species leading to cytotoxic damage to cells. With the specificity of the photosensitizer, PDT is able to localize to tumor cells without damaging nearby healthy tissue [12,53]. 5-aminolevulinic acid (5-ALA), a photosensitizer that is widely used in the clinic due to its ability to image tumors, has proven to be successful in combination with PDT [54]. Based on the finding that CTSE was overexpressed in PDAC, the 5-ALA residue was incorporated into a peptide that is selectively cleaved by CTSE, creating a potent prodrug that can release 5-ALA in a controlled manner in cells that express CTSE like tumor cells [12]. The 5-ALA prodrug selectively causes PDAC cell death *in vitro* and *in vivo* when combined with PDT [12]. In another study, use of the gold nanocluster AuS—U11, improved CLE-guided PDT and photothermal therapy (PTT) by directing uptake of 5-ALA and the fluorescent dye Cy5.5 to cancer cells via CTSE-expression specificity to improve imaging and treatment of cancer cells [55]. This technology allowed for tumor specific near-infrared imaging and treatment [55]. These studies illustrate how technologies could be combined and applied to increase our ability to selectively image and treat cancer cells. Selectivity and specificity can be achieved by taking advantage of our current knowledge regarding the expression of molecules, such as CTSE, in tumor cells that could assist in the development of novel technologies to image and deliver therapeutics.

Cathepsin E expression in other pancreatic diseases

The incidence of pancreatic cysts in the US is estimated at 2—3% in adults, and the detection of cases continues to increase due to a greater usage of high-resolution abdominal imaging [56—58]. Broadly pancreatic cysts can be either premalignant mucinous (intraductal papillary-mucinous neoplasms [IPMN], mucinous cystic neoplasm [MCN]) or non-mucinous cysts. Currently, there is a need for biomarkers that can accurately differentiate between mucinous and non-mucinous cysts and also predict which mucinous (IPMN, MCN) cysts will progress to cancer. CTSE activity has been proposed as a better biomarker to distinguish mucinous from non-mucinous pancreatic cysts when compared to cyst fluid carcinoembryonic antigen (CEA), the commonly used clinical biomarker [59]. In 2002, cDNA -based gene expression profiling of

4992 genes in IPMNs showed 8.4-fold increased expression of CTSE in the IPMNs compared to normal tissue [60]. Moreover, global RNA gene expression profiling found that CTSE was expressed 6.6-fold higher in the neoplastic epithelium of MCNs compared to the normal pancreatic ductal epithelium [61]. In addition to standard of care imaging studies (CT and/or MRI), endoscopic ultrasound needle-guided CLE with molecular analysis of pancreatic cystic fluid has revealed high diagnostic accuracy in differentiating mucinous from non-mucinous pancreatic cysts [62]. The potential addition of CTSE activity and expression to above strategy is a promising methodology to assist in the accurate and early diagnosis of pancreatic cysts that are more susceptible to become malignant and allow for early intervention [63]. This strategy was tested using a preclinical model of PDAC with promising results [11] and sets the premise for clinical studies.

Chronic pancreatitis is difficult to differentiate from PDAC using traditional imaging; however, CTSE is differentially expressed in both, compared to the normal pancreatic ductal epithelium. In a 1996 study, expression of CTSE in the pancreatic fluid was found in 8 of 11 (72.7%) subjects with PDAC and 4 of 43 (9.3%) subjects with chronic pancreatitis [19]. CTSE was later detected in the pancreatic fluid of 16 of 25 (64%) subjects with PDAC but only 6 of 76 (7.9%) subjects with chronic pancreatitis [10]. In both studies, the difference between CTSE detected in pancreatic fluid from subjects with PDAC versus subjects with chronic pancreatitis was significant, suggesting that the expression of CTSE could serve as a biomarker to differentiate between the two diseases [10,19]. Differential expression of CTSE was also observed at the RNA level when pancreas tissue from subjects with CP was compared to those with PDAC [13].

Conclusion

Currently the biological roles of CTSE expression and activity in PDAC, pancreatic cysts, chronic pancreatitis, and other types of cancers remain unclear. The current functions attributed to CTSE in non-pancreatic diseases includes protein turnover, immune response regulation, and apoptosis (Fig. 1). The development of CTSE-specific inhibitors, imaging, and therapeutic probes that leverage its protease function could assist in characterizing CTSE functions in various diseases. The development and use of CTSE-specific imaging could assist future clinical imaging of tumors without the need for invasive procedures (Fig. 2). Similarly, therapeutic probes have the potential to add novel therapeutic options (Fig. 2). The preliminary evidence that CTSE expression and activity can be used as a disease biomarker needs to be validated in a larger data set using well phenotyped subjects with PDAC as well as disease and healthy controls. Validation of the initial observations represents an opportunity to improve patient outcomes in PDAC and other pancreatic diseases (Fig. 3).

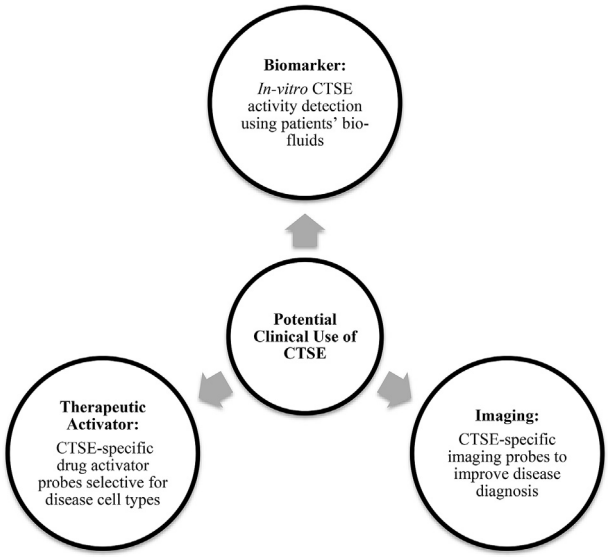

Fig. 2. Potential Clinical Applications of CTSE. Further development of the clinical uses of CTSE could assist in the earlier detection and treatment of PDAC and other pancreatic diseases to improve patient outcomes.

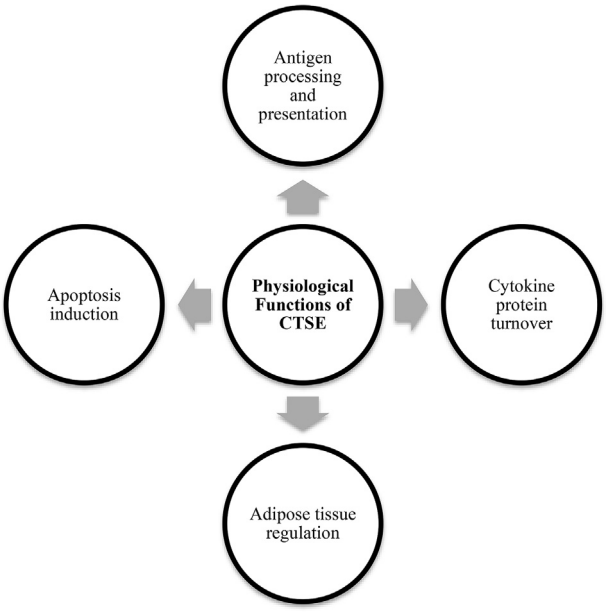

Fig. 1. Cathepsin E Functions: The current functions attributed to CTSE in non-pancreatic diseases include protein turnover, immune response regulation, and apoptosis.

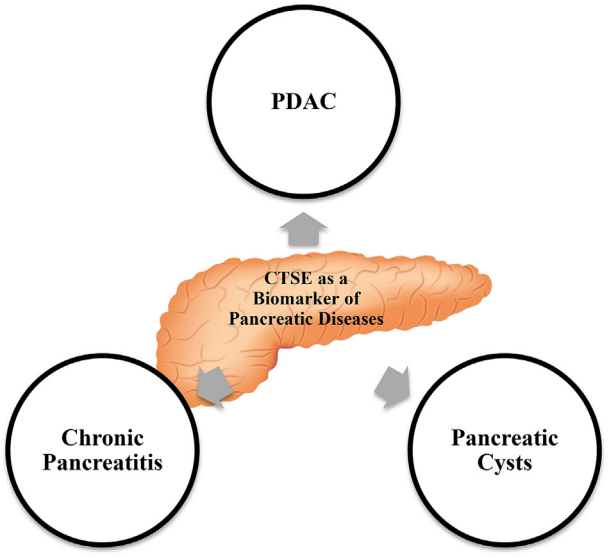

Fig. 3. CTSE Expression is increased in PDAC, CP, and pancreatic cysts. Increased expression and activity of CTSE could serve as a biomarker of various pancreatic diseases.

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Conflicts of interest/disclosures

ZC-M is one of the inventors in Cathepsin E-related patents US9265844 B2 “Protease Degradable Polypeptides and Uses Thereof” and US9439976 B2 “Compositions and Methods for Using Cathepsin E Cleavable Substrates”.

Author contributions

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2. Sabrina Kaul - study concept and design, reviewed literature, analysis and interpretation of data, drafting of initial manuscript, critical revision of the final manuscript, final approval of the version to be published
3. Marcus Hong - study concept and design, reviewed literature, analysis and interpretation of data, drafting of initial manuscript, critical revision of the final manuscript, final approval of the version to be published
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7. Darwin L. Conwell, MD, MS - critical revision of the final manuscript, final approval of the version to be published
8. Zobeida Cruz-Monserrate, PhD - study concept and design, reviewed literature, analysis and interpretation of data, drafting of initial manuscript, critical revision of the final manuscript, final approval of the version to be submitted, and study guarantor

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