REVIEW

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Calreticulin: a potential anti-cancer therapeutic target

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Received February 15, 2017, accepted April 5, 2017

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Pharmazie 72: 503–510 (2017) *doi: 10.1691/ph.2017.7031*

Calreticulin (CRT) is an endoplasmic reticulum luminal calcium-binding protein with multiple cellular functions, including intracellular Ca^{2+} homeostasis, oxidative stress responses, and lectin binding. CRT can also modulate cell adhesion, cell-cell interactions, migration, phagocytosis, integrin-dependent Ca^{2+} signaling, and immune responses, and plays an important role in cellular proliferation, differentiation, and apoptosis. Given these roles, it is not surprising that CRT function has important implications in health and disease. Considerable evidence in recent years suggests that CRT dysfunction is associated with cancer and that CRT could be a diagnostic marker and a target for cancer therapy. These topics are discussed in depth in this review.

1. Introduction

Calreticulin (CRT) is an endoplasmic reticulum (ER) luminal calcium-binding protein with multiple functions in physiological and pathological cell processes. Considerable evidence in recent years has suggested that CRT dysfunction plays a role in cancer through its role in cell proliferation, adhesion, invasion, migration, differentiation, apoptosis, phagocytosis, and immunity. In this review, we discuss the function of CRT as well as its potential utility as a cancer diagnostic marker and a therapeutic target.

Abbreviations:

AML	Acute Myeloid Leukemia
CALR	Calreticulin gene
CBFB-SMMHC	Core-binding factor β-smooth muscle myosin heavy chain
CD	Cluster of differentiation
CEBPA	CCAAT/enhancer-binding protein alpha
CRT	Calreticulin
CTL	Cytolytic T Lymphocytes
DADS	Diallyl disulfide
DCs	Dendritic cells
DR5	Death receptor 5
ER	Endoplasmic Reticulum
FasL	Fas ligand
HCC	Hunmanhepatocarcinoma cell
HCT	Human Colorectal
HLA-G	Human leucocyte antigen-G
HMGB	High-mobility group box
HPV	Human papillomavirus
HTLV-1	Human T-cell leukemia/lymphoma virus type 1
KDEL	Lysine-aspartate-glutamate-leucine
LRP	Lipoprotein receptor-related protein
MCF	Michigan Cancer Foundation
MDCK	Madin-Daby canine kidney cells
MHC	Major histocompatibility complex
NBs	Neuroblastomas
NBT	Nitroblue tetrazolium
NK	Natural killer
OSCC	Oral Squamous Cell Carcinoma
PC	Pancreatic cancer
PDT	Photodynamic therapy
PI3K	Phosphoinositide 3-kinase
PS	Phosphatidylserine
siRNA	Small interfering RNA
TcCRT	Trypanosoma cruzi CRT
TSP	Thrombospondin
UICC	Union for International Cancer Control

2. Structure and function of CRT

CRT was first identified in 1974 as a Ca^{2+} storage protein in muscle sarcoplasmic reticulum (Opas et al. 1991). However, subsequent work demonstrated that CRT is abundant in non-muscle tissues (Krause et al. 1997). CRT consists of three domains; the N-, P-, and C-domain, and contains a KDEL (lysine-aspartate-glutamate-leucine) ER retention peptide and a signal sequence at the C- and N-termini, respectively. Each domain has a putative function (Lu et al. 2015). The structure of CRT is highly conserved through evolution. The protein affects store-operated Ca^{2+} entry and influences Ca^{2+}-dependent transcriptional pathways during embryonic development (Pinto et al. 2013). In catfish, three CRT genes are widely expressed in various tissues under homeostatic conditions, and their expression is significantly upregulated following infection and/or changes in iron levels (Liu et al. 2011). In the Chinese mitten crab, CRT (EsCrt) is expressed in the gill, hepatopancreas, hemocytes, and intestine, and its levels are altered by challenge with lipopolysaccharides, peptidoglycans, *Staphylococcus aureus*, and *Vibrio parahaemolyticus*. EsCrt thus appears to be involved in anti-bacterial immunity (Huang et al. 2016). In shrimp, CRT is thought to play important roles in Ca^{2+} homeostasis, chaperoning, and immune function (Luana et al. 2007). Mammalian CRT is a 46-kDa protein located inside and outside the ER (Michalak et al. 2009), but it is localized at the ER in most cell types (Opas et al. 1991). In the ER lumen, CRT functions as a regulator of intracellular Ca^{2+} homeostasis, oxidative stress responses, and lectin binding, and all of these functions are affected by the continuous fluctuations in Ca^{2+} concentrations within the ER (Liu et al. 2011). CRT affects intracellular Ca^{2+} homeostasis by modulating ER Ca^{2+} storage and transport. It is a highly versatile lectin-like chaperone, and it participates in the synthesis of a variety of molecules, including ion channels, cell surface receptors, integrins, and transporters (Michalak et al. 1999). It is also involved in ensuring proper folding of newly synthesized proteins and glycoproteins and, together with calnexin and ERp57, constitutes the "CRT/calnexin cycle" that is responsible for quality control pathways in the ER (Kawabe et al. 2010). CRT has also been found outside of the ER compartment,

including in other cytosolic membrane-bound organelles as well as in the extracellular matrix, where it exerts a number of physiological and pathological effects (Johnson et al. 2001). CRT also localizes to the nuclear envelope, the nucleus, and nucleoli-like structures in some cell types, including cytolytic T lymphocytes (CTL), and in acrosomal vesicles of sperm cells (Dupuis et al. 1993; Nash et al. 1994; Sørensen et al. 2004). Cell surface CRT can modulate cell adhesion, cell-cell interaction, migration, phagocytosis, integrin-dependent Ca^{2+} signaling, and immunity (Luana et al. 2007; Gold et al. 2010; Zamanian et al. 2013), and also plays an important role in cell proliferation, differentiation, and apoptosis (Sun et al. 2015; Pallero et al. 2008). CRT dysfunction is accompanied to a number of diseases (Ni et al. 2007; Wang et al. 2012), and it regulates an array of cellular responses in pathological processes, such as wound healing, fibrosis, and cancer (Gold et al. 2010). Very recently, mutation-specific immunohistochemistry has identified mutations in CRT in myeloproliferative neoplasms (Sun et al. 2015; Clinton et al. 2016; Andrici et al. 2016). CRT dysfunction has been associated with several cancers (Sun et al. 2015), and study results support its role in tumor formation and progression, depending on the cell type and clinical disease stage (Lu et al. 2015; Zamanian et al. 2013). Recent findings suggest that CRT could be a target for the development of novel therapeutics (Čiplys et al.2015).

3. Expression and translocation of CRT in cancer

In mammals, the expression level of CRT differs markedly in various organs and tissues, suggesting that CRT plays a specific role in each cell type. Proteomic analysis has been useful in examining the expression of cancer associated candidate proteins, including CRT (Alfonso et al. 2005). While CRT is minimally expressed in most normal cells, high CRT expression levels have been observed in several cancers (Chao et al. 2010). It is upregulated in high-degree microsatellite instability colorectal adenocarcinomas, squamous cell carcinoma of the esophagus tumor tissue (Banerjea et al. 2004; Jazii et al. 2006) and also highly expressed in oral squamous cell carcinoma (OSCC) cell lines, breast cancer cell lines MCF-7 and MDA-MB-231 (Chiang et al. 2013; Prathyuman et al. 2010; Chen et al. 2015). Levels of CRT mRNA were also significantly higher in AML than in other hematologic malignancies (Park et al. 2015). Many research results suggested that the CRT level in pancreatic cancer, bladder cancer and infiltrating ductal breast carcinoma tissues might correlate with carcinogenesis and cancer progression (Kageyama et al. 2004; Chen et al. 2007; Lu et al. 2011; Kabbage et al. 2013; Sheng et al. 2014). CRT expression was also higher in the more aggressive MDA-MB-231 cells compared with MCF-7 cells at both the mRNA and protein levels. Moreover, its expression was positively correlated with both the tumor size and development of distant metastasis (Lwin et al. 2010). CRT concentrations in the serum of lung cancer patients were higher than in healthy individuals, and the level of CRT on lung cancer cell membranes was associated with tumor pathological classification and grade (Liu et al. 2012). Among 68 neuroblastomas (NBs) tested, 32 (47.1%) were positive for CRT, and CRT expression correlated with improved survival (Hsu et al. 2005). The level of CRT was higher in neuroglioma H4 cells than in glioblastoma cells (U251MG and T98G), and correlated well with sensitivity to γ-irradiation (Okunaga et al. 2006). CRT was highly expressed in nude mice bearing tumors derived from subcutaneous injection of the human colorectal cell line HCT116 (Wang et al. 2006).

While most studies have observed that CRT expression is increased in cancer tissues, downregulation has also been observed in laryngeal squamous cell carcinoma lesions, cervical carcinomas, human colon adenocarcinomas, human colonic cancer cells, small cell lung cancer line (H1339) and a lung adenocarcinoma line (HCC) (Ogino et al. 2006; Toquet et al. 2007; Mehta et al. 2008; Bergner et al. 2009). Also, the expression of CRT was 10-fold lower in mice with advanced prostate cancer compared with normal mice (Ruddat et al. 2005). After a mean follow-up of 3.6 years, survival was significantly worse in bladder carcinoma patients with a lower CRT score (Cathro et al. 2010). CRT expression in different tumor tissues/cells (Table).

Table: CRT expression in different tumor tissues/cells

	Tumor tissues	Tumor cells
Upregulated	colorectal adenocarcinomas esophagus tumor tissue the serum of lung cancer pancreatic cancer bladder cancer infiltrating ductal breast carcinomas	oral squamous cell carcinoma cell line breast cancer cell line (MCF-7) breast cancer cell line (MDA-MB-231) neuroblastomas (NBs) neuroglioma H4 cell line (U251MG) glioblastoma cell line (T98G) human colorectal cell line (HCT116)
Downregulated	laryngeal squamous cell carcinomas cervical carcinomas human colon adenocarcinomas prostate cancer bladder carcinomas	human colonic cancer cell line small cell lung cancer line (H1339) lung adenocarcinoma line (HCC)

CRT is usually not found in the ER lumen in cancer cells, but on the cell surface of breast cancer and acute myeloid leukemia (AML) cells (Mans et al. 2012; Ramesh et al. 2016). It was also detectable in the cytoplasm of the breast cancer lines MDA-MB-231 and MCF-7 (Lwin et al. 2010). CRT was additionally present in the nuclear matrix of colon cancer tissue and may be related to increased cell growth and tumorigenesis (Yoon et al. 2000; Brünagel et al. 2003).

Treatment of cancer cells with fenretinide, idazoxan hydrochloride, 7,8-diacetoxy-4-methylcoumarin, diallyl disulfide (DADS) can affect the expression of CRT (Corazzari et al. 2007; Eilon et al. 2009; Verma et al. 2011; Yi et al. 2016). Meantime, various reagents such as doxorubicin, mitoxantrone, anthracyclines, wogonin and anthracycline also can affect the location of CRT in tumor cells (Tufi et al. 2008; Cao et al. 2009; De Boo et al. 2009; Mans et al. 2012; Yang et al. 2012).

Besides, mutations in the CRT gene (CALR) were recently identified in approximately 70–80% of patients with JAK2-V617F–negative essential thrombocytosis and primary myelofibrosis. Mutant CRT-expressing cells induced monocyte hyperreactivity through a paracrine mechanism (Garbati et al. 2016). During embryogenesis, certain stimuli may induce overexpression of CRT in the mice heart and alter numerous signaling pathways, subsequently inducing pathology (Martinho-Dias et al. 2016). Photodynamic therapy significantly protected against a subsequent challenge with live CT26 cells, and this protection was inhibited by siRNA for CRT (Tanaka et al. 2016).

4. CRT effects on the proliferation of cancer cells

Manipulation of CRT levels profoundly affects malignant cell proliferation (Lu et al. 2015; Zamanian et al. 2013). CRT has been shown to exert anti-angiogenic activity, promote tumor lymphocyte infiltration, and inhibit tumor growth (Wang et al. 2012). In gastric cancer cells, the proliferation rate of CRT-overexpressing cells was higher and that of CRT-knockdown cells was lower than the proliferation rate of control cells, suggesting that CRT might play an essential role in facilitating gastric cancer cell proliferation (Chen et al. 2007). Stable knockdown of CRT in OSCC cells resulted in significantly reduced growth rate, colony-forming capacity, and anchorage-independent growth compared with control cells (Chiang et al. 2013). Similarly, knockdown of CRT suppressed J82 bladder cancer cell proliferation (Lu et al. 2011). Overexpression of CRT was shown to upregulate the expression and secretion of placental growth factor, vascular endothelial growth factor, and tissue growth factor; enhance angiogenesis; and facilitate prolif-

eration of gastric cancer cells, consistent with the association of CRT with survival in gastric cancer patients (Chen et al. 2009). Overexpression of CRT in MCF-7 and MDA-MB-231 cells significantly suppressed cell viability. The combination thrombospondin (TSP) treatment and overexpression of CRT significantly inhibited the growth of MCF-7 cell xenografts (Chen et al. 2015). Similarly, overexpression of CRT suppressed proliferation of the NB cell line stNB-V1 (Weng et al. 2015). *Trypanosoma cruzi* CRT (TcCRT) displayed remarkable anti-angiogenic properties, and C57BL/6 mice were immunized with TcCRT showed tumor growth impaired (Aguilar-Guzmán et al. 2014). siRNA-mediated knockdown of CRT expression significantly inhibited the proliferation of DADS-treated HL-60 cells (Yi et al. 2016). In pancreatic cancer cells, CRT regulates cell proliferation and other behavior in a MEK/ERK pathway-dependent manner. The interaction between CRT and the MEK/ERK pathway could provide a novel idea for revealing the malignant biology of PC (Sheng et al. 2014). The human T-cell leukemia /lymphoma virus type 1 (HTLV-1) p12I protein interactions with CRT and calnexin may contribute to the survival and proliferation of infected T cells in the HTLV-1-infected patients (Fukumoto et al. 2009). CRT profoundly affects the wound healing process by stimulating cell growth and increasing extracellular matrix production (Gold et al. 2006). Moreover, CRT translocation and expression of mutant CRT also affect tumor cell proliferation. In AML cells, intracellular CRT translocated to the cell surface, suggesting a role in tumor suppression (Mans et al. 2012). Mutant CRT causes myeloproliferation through activation of the JAK–STAT pathway, similar to the effect of the V617F mutation in JAK2 (Daitoku et al. 2016).

5. CRT is involved in tumor cell adhesion, invasion, migration, and cell–cell interactions

CRT is an essential modulator of cell adhesive functions and integrin-initiated Ca^{2+} signaling (Coppolino et al. 1997). Overexpression of CRT correlates with increased cell adhesiveness. These changes may be due to CRT-mediated effects on a signaling pathway from the ER, which impinges on the Wnt signaling pathway via the cadherin/catenin protein system and involves changes in the activity of protein tyrosine kinases and/or phosphatases (Fadel et al. 2001). The anti-adhesive activity of TSP is mediated by the N-terminal domain of cell surface CRT. TSP induces restructuring of focal adhesions through binding of amino acids 17–35 (hep I peptide) of TSP to a cell surface form of CRT (Goicoechea et al. 2002). The N-terminal domain of TSP1 binds to the CRT–low-density lipoprotein receptor-related protein (LRP1) receptor co-complex to signal downregulation of cell adhesion and increase cell motility through focal adhesion disassembly (Pallero et al. 2008). In addition to their role in immunogenicity and tumorigenesis, interactions between CRT and integrins have been described during cell adhesion, which is an essential process for cancer metastasis (Lu et al. 2015). CRT is also involved in the progression of cancer invasion and migration (Zamanian et al. 2013). siRNA-mediated knockdown of CRT expression significantly decreased invasiveness of DADS-treated HL-60 cells, clearly demonstrating a role for CRT in cell invasion (Yi et al. 2016). CRT is overexpressed in various cancers. Overexpression of CRT enhanced, whereas CRT knockdown suppressed, cell migration and attachment by J82 bladder cancer cells. Moreover, tumors derived from J82 CRT-RNAi cells were significantly smaller and had fewer metastatic sites in the lung and liver *in vivo* than did vector-transfected control cells (Lu et al. 2011). In addition, compared with CRT-knockdown cells, CRT overexpression in the human gastric cancer cell line AGS increased their migration rate, suggesting that it could be an important therapeutic target for this disease (Chen et al. 2007). CRT overexpression enhanced angiogenesis and migration of gastric cancer cells, which is consistent with the association of CRT with microvessel density, tumor invasion, and lymph node metastasis in gastric cancer patients (Chen et al. 2009). Furthermore, CRT-overexpressing Madin-Darby canine kidney cells showed enhanced migration through Matrigel-coated Boyden chamber wells compared with control cells (Hayashida et al. 2006). Overexpression of CRT in MCF-7 and MDA-MB-231 cells significantly suppressed cell migration (Chen et al. 2015). The interaction between CRT and MEK/ERK pathway regulates cell migration and invasion in pancreatic cancer (Sheng et al. 2014). CRT also affects cell-cell interactions of cancer cells. CRT regulates the epithelial–mesenchymal transition-like change in cellular phenotype by modulating the Slug/E-cadherin pathway, suggesting a novel function of CRT in the cell-cell interactions of epithelial cells (Hayashida et al. 2006). CRT is stored in the cytotoxic granules of CTLs and natural killer (NK) cells and is released with granzymes and perforin upon recognition of target cells. CRT was required for efficient CTL–target cell interaction and formation of the death synapse (Sipione et al. 2005) and enhanced the expression of adhesion molecules, such as intercellular adhesion molecule and vascular cell adhesion molecule, on tumor endothelial cells. This upregulated expression resulted in enhanced leukocyte– endothelial cell interactions and increased lymphocyte infiltration into tumors (Wang et al. 2012).

6. CRT is an essential factor for tumor cell differentiation

CRT plays an important role in cellular differentiation (Sun et al. 2015; Wang et al. 2012). CRT is highly expressed in dedifferentiated liposarcoma cells. Downregulation of CRT by siRNA induced adipogenesis of dedifferentiated liposarcoma cells and reduced cell proliferation. Thus, aberrantly expressed CRT in dedifferentiated liposarcoma was involved in its dedifferentiation and/or tumor progression (Hisaoka et al. 2012). Introduction of the CRT gene into H9c2 rat cardiomyoblasts significantly suppressed protein kinase B/Akt signaling during cell differentiation, suggesting that CRT plays an important role in cardiac differentiation (Kageyama et al. 2002). Studies on CRT-deficient and transgenic mice have revealed that CRT may be an upstream regulator of the Ca^{2+}-dependent pathways that control cellular differentiation and/or organ development (Michalak et al. 2002). Additional studies have suggested that CRT may be an important regulator of the differentiation and function of mouse bone marrow-derived mast cells (Ryu et al. 2012). Ablation of CRT diminished adiponectin-stimulated cyclooxygenase 2 expression and endothelial cell differentiation (Ohashi et al. 2009). Moreover, several reports have demonstrated that manipulation of CRT levels profoundly affects cancer cell differentiation (Lu et al. 2015). CRT is essential for the differentiation of NB cells, suggesting that CRT may be a useful marker for managing the treatment of NB patients (Hsu et al. 2005). The c-Jun-NH (2) kinase–CRT-dependent pathway is essential for neuronal differentiation elicited by Notch signaling blockade (Chang et al. 2010). Overexpression of CRT enhanced the differentiation of stNB-V1 cells, and vascular endothelial growth factor-A was involved in CRT-related differentiation of NB cells (Weng et al. 2015). CRT expression was significantly associated with the mucinous differentiation of human colonic adenocarcinomas; thus, CRT is likely to play a pivotal role in the differentiation of this tumor (Toquet et al. 2007). The myeloid transcription factor CCAAT/enhancer-binding protein alpha (CEBPA) is crucial for normal granulopoiesis. CRT, an inhibitor of CEBPA translation, was induced at the mRNA and protein levels in core-binding factor β–smooth muscle myosin heavy chain (*CBFB-SMMHC*)-positive AML patients and after expression of CBFB-SMMHC in the U937 cell system. These results suggested that modulation of CEBPA by CRT might be a novel mechanism involved in the differentiation block in *CBFB-SMMHC* AML (Helbling et al. 2005). CRT was strongly activated after induction of the leukemic fusion gene AML1–MDS1–EVI1 (AME) in a cell line and in AME patient samples. Moreover, inhibition of CRT by siRNA restored the CEBPA levels (Helbling et al. 2004). siRNA-mediated knockdown of CRT expression significantly increased the expression of CD11b and reduced the expression of CD33 in DADS-treated HL-60 cells. Nitroblue tetrazolium (NBT) assays showed decreased NBT reduction in cells overexpressing CRT and increased NBT reduction in the CRT

siRNA group, clearly supporting a role for CRT in the differentiation of DADS-treated HL-60 cells (Yi et al. 2016). Vasostatin (the N-terminal domain of CRT) is released from cell surface CRT and has been shown to impair differentiation of myeloid cells and vascularization of the bone marrow microenvironment (Mans et al. 2012).

7. CRT plays an important role in cancer cell apoptosis

Among the many cellular processes regulated by CRT is cell death and recent reports indicate that CRT plays a role in the modulation of cancer cell apoptosis. Human breast cancer MCF-7 cells transfected with CRT were significantly more susceptible to apoptosis than control cells, suggesting a novel mechanism by which cancerous cells can be triggered to die (Prathyuman et al. 2010). Overexpression of CRT in MCF-7 and MDA-MB-231 cells significantly promoted cell apoptosis (Chen et al. 2015). Thus, CRT is involved in the regulation of radiosensitivity and radiation-induced apoptosis in malignant glioma cells (Okunaga et al. 2006). Overexpression of CRT also promoted differentiation-dependent apoptosis in H9c2 cells by suppressing the Akt signaling pathway (Kageyama et al. 2002). CRT can bind to Fas ligand (FasL) and inhibit Fas/FasL-mediated apoptosis of Jurkat T cells. Increased CRT also inhibited Fas/FasL-mediated neuronal cell apoptosis during the early stage of ischemic stroke, suggesting that it may have a protective role soon after ischemia-reperfusion injury (Chen et al. 2015). In gemcitabine- or oxaliplatin-treated Capan-2 cells, a human pancreatic adenocarcinoma line, CRT knockdown significantly decreased phospho-ERK expression and chemoresistance independently of activated p53 and caspase-3-related apoptosis (Sheng et al. 2014). CRT is associated with induction of cancer cell apoptosis by irradiation and a number of cytotoxic reagents. Garlic compounds induced apoptosis in glioblastoma cells through expression of CRT (Das et al.2007). Zearalenone can induce apoptosis of human leukemic cells HL-60 and U937 via induction of ER stress. In HL-60 cells, this effect was accompanied by a change in CRT levels, indicating that CRT may be involved in the induction of apoptosis (Banjerdpongchai et al. 2010). CRT exposure was required for the immunogenicity of γ-irradiation- and ultraviolet C light-induced apoptosis (Obeid et al. 2007). The development of paroxysmal nocturnal hemoglobinuria is associated with the accumulation of CRT mutations that create an intrinsic survival benefit for clonal expansion (Fraiman et al. 2016). TSP1 signaling through CRT–LRP1 activates cell survival signals such as PI3K activation (Pallero et al. 2008). In response to some chemotherapeutic agents such as anthracyclines and oxaliplatin, cancer cells undergo immunogenic apoptosis. One of the peculiarities of immunogenic apoptosis is the early cell surface exposure of CRT, which is necessary and sufficient to increase pro-immunogenic killing by other chemotherapies. Cell death induced by anthracyclines, oxaliplatin, and γ-irradiation is causally connected to the exposure of CRT on the tumor cell surface before apoptosis occurs. The CRT exposure pathway is activated by pre-apoptotic ER stress, which results in CRT translocation to the plasma membrane (Obeid et al. 2007; Zitvogel et al. 2010).

8. CRT is associated with tumor cell phagocytosis

High expression of CRT on the surface of human cancer cells acts as a pro-phagocytic signal. Increased CD47 expression correlates with CRT expression on cancer cells and is necessary for protection against CRT-mediated phagocytosis (Chao et al. 2010). As a specific ligand, CRT on the surface of apoptotic cells could mediate recognition and clearance of apoptotic cells by phagocytes (Cao et al. 2009). CRT was the first molecule to be identified as a marker for phagocytosis of apoptotic cells by *Drosophila* phagocytes (Kuraishi et al. 2007). Cell surface CRT is known to transduce pro-phagocytic signals to macrophages and has been shown to be an important regulator of macrophage engulfment (Daitoku et al. 2016). CRT on the cell surface of living and dying cells promotes phagocytic uptake (Raghavan et al. 2013). Cell surface CRT is considered a pro-phagocytic signal that promotes uptake of cancer cells by immune cells (Lu et al. 2015). Mutant CRT adversely affects myeloid cells throughout their lifespan, from the production of critical glycoproteins in bone marrow precursors to the clearance of spent neutrophils (Nauseef et al. 2016). N-terminal arginylation of the ER chaperone BiP/GRP78, protein disulfide isomerase, and CRT is co-induced with autophagy during innate immune responses to cytosolic foreign DNA or proteasomal inhibition, and is associated with increased ubiquitylation (Cha-Molstad et al. 2015). Overexpression of CRT in MCF-7 and MDA-MB-231 cells has no effect on autophagy, but interestingly, the combination of TSP treatment and CRT overexpression significantly induced autophagy in MCF-7 xenografts (Chen et al. 2015). In vitro recombinant TcCRT bound to melanocytes, promoting the incorporation of human C1q and subsequent macrophage phagocytosis of tumor cells (Aguilar-Guzmán et al. 2014). CRT acts as a second general recognition ligand by binding and activating LRP on the engulfing cell. The apoptotic cell creates an environment where "don't eat me" signals are rendered inactive and "eat me" signals, including CRT and phosphatidylserine (PS), congregate together and signal for removal (Gardai et al. 2005). CRT presentation on the cell surface is an important hallmark of immunogenic cell death, serving as the pro-phagocytic signal for macrophages (Liu et al. 2016). The phospholipid-binding site of CRT is a key anchor point for the cell surface expression of CRT on apoptotic cells, and CRT acts as a PS-bridging molecule that cooperates with other PS-binding factors to promote the phagocytosis of apoptotic cells (Wijeyesakere et al. 2016). In the process of tumor cell apoptosis induced by specific stimuli, CRT rapidly translocates from the ER to the cell membrane. As a specific ligand, CRT on the surface of apoptotic tumor cells could mediate the recognition and clearance of apoptotic tumor cells by professional and non-professional phagocytes (Wu et al. 2013). CRT has a multifaceted role in carcinogenesis.

9. CRT acts as a cancer diagnostic marker and anti-cancer therapeutic target

CRT could serve as a biomarker to predict therapy-associated immune responses, and tactics to expose CRT might improve the clinical efficacy of many cancer therapies (Obeid et al. 2007). CRT present on the surface of colonic and breast cancer cells could be a useful biomarker for these cancers (Ramesh et al. 2016). Multivariate analysis demonstrated that CRT expression is an independent prognostic factor in patients with advanced-stage NB (Hsu et al. 2005). CRT is also a potential biomarker for, and may contribute, to the malignant phenotypes of OSCC cells (Chiang et al. 2013) and could be a diagnostic and prognostic biomarker in lung cancer (Liu et al. 2012). Furthermore, detection of autoantibodies to CRT isoforms may have utility for the early diagnosis of pancreatic cancer (Hong et al. 2004). The high occurrence and specificity of serum anti-CRT autoantibodies in the majority of patients with some gastrointestinal malignancies provide evidence for their possible clinical relevance (Pekáriková et al. 2010). Urinary CRT may be useful for the diagnosis of bladder urothelial cancer. Concomitant use of CRT, γ-synuclein, and soluble catechol-o-methyltransferase had higher sensitivity for detection of bladder cancer than did CRT alone. Alteration of CRT expression levels might affect bladder cancer progression in vitro and in vivo (Kageyama et al. 2004; Lu et al. 2011; Iwaki et al. 2004; Kageyama et al. 2009). Further studies of CRT may be helpful in finding new drug targets for cancer chemotherapy (Wang et al. 2006); for example, the interaction between CRT and the MEK/ERK pathway might provide new ideas for gene-targeted chemotherapy in pancreatic cancer (Sheng et al. 2014). Mouse melanoma cells coated with a recombinant fusion protein of mouse CRT and virus G protein-coupled receptor induced specific anti-tumor immunity though the activation of dendritic cells (DCs). These results may provide an experimental basis for the development of new tumor vaccines (Wu et al. 2013). Combined vaccination with CRT/human papillomavirus (HPV) E6 and CRT/E7 DNA generated significantly better therapeutic anti-tumor effects

against E6- and E7-expressing tumors than did vaccination with either CRT/E6 DNA or CRT/E7 DNA alone (Peng et al. 2006). Vasostatin is an anti-angiogenic chemotherapy, and delivery of vasostatin by recombinant pseudotype adeno-associated virus 2/5 has been used as a gene therapy approach for lung cancer (Jazowiecka-Rakus et al. 2006; Cai et al. 2008). Depending on the signal transduction pathway, tumor cells responding to chemotherapy or radiotherapy can express 'danger' or 'eat me' signals, such as CRT, on the cell surface (Tesniere et al. 2008). Vaccination with a human CRT-E6-E7-L2 DNA vaccine induced a potent E6/E7-specific CD8+ T cell immune response, resulting in a significant therapeutic effect against E6/E7-expressing tumor cells (Kim et al. 2008). The MHC CIITA is a master regulator of MHC class II expression and also induces expression of class I molecules. The combination of CIITA DNA with CRT/E6 and the invariant chain (Ii) linked to the pan HLA-DR-reactive epitope (Ii-PADRE) DNA vaccines represents a potentially effective means to combat tumors in the clinical setting (Kim et al. 2008). Tumors that possess an intrinsic defect in the CRT-translocating machinery become resistant to anthracycline chemotherapy due to their incapacity to elicit an anti-cancer immune response (Panaretakis et al. 2008). Treatment of tumor-bearing mice with chemoimmunotherapy, combining cisplatin and CRT/E7 DNA, generated the highest E7-specific CD8+ T cell immune response and produced the greatest anti-tumor effects (Tseng et al. 2008). The combination of bortezomib and CRT/E7 generated more potent E7-specific CD8+ T cell immune responses and better therapeutic effects against TC-1 tumors in tumor-bearing mice compared to monotherapy (Tseng et al. 2008). Death receptor 5 (DR5) and CRT/E7 administered *via* gene gun resulted in further enhancement of the E7-specific immune response and anti-tumor effects (Tseng et al. 2008). CRT-mediated acetylation could hold a key to the design of drugs targeting protein acetylation for improving cancer therapy (Dwarakanath et al. 2008). Anti-cancer activity of targeted proapoptotic peptides and chemotherapy is greatly improved by targeted cell surface CRT-inducer peptides (Obeid et al. 2009). 7, 8-Diacetoxy-4-methylcoumarin-induced death of human tumor cells is influenced by CRT. Thus, targeting the CRT transacetylase system may be an attractive approach for increasing the efficacy of anti-cancer therapies (Verma et al. 2011). Blockade or knockdown of CRT suppressed the phagocytosis of anthracycline-treated tumor cells by DCs and abolished their immunogenicity in mice. These data identify CRT as a key feature determining anti-cancer immune responses and delineate a possible strategy for immunogenic chemotherapy (Obeid et al. 2007). In tumor vaccine models, drugs that induced cell surface CRT conferred enhanced tumor protection in an extracellular CRT-dependent manner (Raghavan et al. 2013). Thus, CRT treatment can render tumor cells more vulnerable to immunotherapy and improve the therapeutic efficacy of immunotherapy (Wang et al. 2012). Pre-apoptotic exposure to CRT is required for immunogenic cell death. PDT with glycoconjugated chlorin induced immunogenic cell death, and this effect was directed by CRT expression and translocation (Tanaka et al. 2016). Human cancers that are incapable of activating the CRT exposure pathway are refractory to the immune-mediated component of anti-cancer therapies (Zitvogel et al. 2010). The most efficient anti-tumor treatments are those that induce immunogenic cell death, which strongly depends on the quantity of CRT exposed at the cell membrane after immunogenic treatment. ERp57 serves as a new molecular marker of immunogenicity and as a key protein that controls immunogenicity by controlling CRT exposure (Obeid et al. 2008).

10. CRT may play a role in host immunity

CRT has several functions in the immune response. CRT has been shown to bind to peptides brought into the ER by antigen processing-associated transporters (Spee et al. 1997). CRT preparations purified from tumors elicit specific immunity to the tumor from which the CRT was isolated. The specificity of this effect was attributed to the peptides associated with the CRT molecule. CRT molecules can be complexed *in vitro* to unglycosylated peptides and used to elicit a peptide-specific CD8+ T cell response exogenous administration (Basu et al. 1999). CRT acted as an adjuvant to promote DC maturation, which induced CTL development and enhanced MAGE-A3-specific CTL cytotoxicity against non-small cell lung cancer (Liu et al. 2016). Under defined conditions during inflammation, CRT released from neutrophils not only induces an antigenic reaction but also interferes with C1q-mediated inflammatory processes (Kishore et al. 1997). Dying tumor cells can elicit a potent immune response by exposing the CRT/ERp57 complex on the cell surface, even before the cells manifest any signs of apoptosis (Panaretakis et al. 2009). CRT deficiency might be involved in the low HLA-G surface expression of 5-aza-2′-deoxycytidine-treated OCM-1A melanoma cells (Yan et al. 2005). Proteomic analyses of anthracycline-treated tumor cells have recently revealed the critical involvement of CRT in mediating the immunogenicity of dying tumor cells (Apetoh et al. 2007). The pre-apoptotic translocation of intracellular CRT to the plasma membrane surface is critical for immunogenic cell death (Obeid et al. 2007). In the ER, CRT facilitates the folding of major histocompatibility complex (MHC) class I molecules and their assembly factor tapasin, thereby influencing antigen presentation to CTL (Pinto et al. 2013; Raghavan et al. 2013). CRT also participates in the reactions yielding assembly of peptides onto nascent MHC class I molecules. CRT-bound peptides can be re-presented on DC class I molecules for recognition by CD8+ T cells (Nair et al. 1999). MHC class I molecules expressed in a CRT-deficient cell line (K42) assembled normally with β2-microglobulin (Gao et al. 2002). CRT-deficient mice displayed a lower threshold of T cell receptor activation, resulting in enhanced secretion of inflammatory cytokines (Porcellini et al. 2006). Recent research has demonstrated that a CRT homolog from disk abalone (AbCALR) can be stimulated by pathogenic signals, and thus might play a role in host immunity (Udayantha et al. 2016).

Acknowledgements: The present study was sponsored by the National Natural Scientific Foundation of China (81400117), the Hunan Provincial Natural Science Foundation of China (2015JJ4042), China Postdoctoral Science Foundation (2014M562115), Hunan Provincial Postgraduate Students Scientific Research Innovation Project (CX2016B473) and the returned personnel Initial Funding of University of South China (2014XQD46).

Conflicts of interest: None declared.

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