

CALR and *CD47*: An Insight into their Roles in the Disease Progression of MDS and MPN

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Abstract

Myelodysplastic syndrome and myeloproliferative neoplasms are clonal myeloid disorders arising from haematopoietic stem cells that have the tendency to progress into acute myeloid leukaemia. Multiple prognostic scoring systems have been proposed and utilised in clinical practice to predict disease evolution, however none of them can predict treatment response. In solid tumours, the relationship between the pro-phagocytic calreticulin and the anti-phagocytic *CD47* is repeatedly investigated. Overexpression of calreticulin has been documented to produce a pro-phagocytic signal in solid tumour and it is often counteracted by a concomitant expression of the anti-phagocytic *CD47* as they act in response to one another, reflecting an apoptosis vs survival mechanism in response to chemotherapy. The role of both calreticulin and *CD47* are currently poorly understood in myeloid malignancies including myelodysplastic syndrome and myeloproliferative neoplasms. The aim of this review is to elaborate on the current understanding round the roles and implications of calreticulin and *CD47* signalling with in solid and haematological cancers, discuss potential roles for calreticulin and *CD47* expression in transformation of myeloid cells in patients with MDS or MPN into AML and how these advances are starting to be used to design new therapeutic strategies to determine disease progression and treatment response in both solid cancer and myeloid malignancies.

Keywords: Haematological cancers; Solid cancers; Myelodysplastic syndrome; Myeloproliferative neoplasms; Acute myeloid leukaemia

Abbreviations: MDS: Myelodysplastic Syndrome; MPN: Myeloproliferative Neoplasms; *CALR*: Calreticulin; ET: Essential Thrombocythemia; PV: Polycythaemia Vera; MF: Myelofibrosis

Introduction

Myelodysplastic syndromes (MDS), Myeloproliferative neoplasms (MPN) and acute myeloid leukaemia (AML) are myeloid malignancies which are a group of bone marrow diseases arising from clonal disorders of haematopoietic stem cells. MDS and MPN show tendencies to develop into AML as these diseases progress [1]. These diseases originate from a range of genetic events in the hematopoietic stem cells that lead to dysmyelopoiesis, abnormal differentiation, increased apoptosis with ineffective haematopoiesis, cytopenias or leucocytosis [2,3]. Scoring systems for prognosis of the disease are mainly based on bone marrow/peripheral blood features, cytogenetics and some clinical manifestations, with no specific genetic marker highlighting subtypes or stages of these conditions or predicting transformation and disease progression. In solid tumours, characterisation of the relationship between the pro-phagocytic calreticulin (*CALR*) and the anti-phagocytic *CD47* markers is starting to show they act in response to one another, reflecting a possible apoptosis vs survival mechanism in response to chemotherapy. It has been suggested that calreticulin and *CD47* could also play a similar role within myeloid malignancies, however their role within these diseases has been less well established. In this review using leading published data (searched for on Pubmed and Google scholar) we will discuss the current challenges in disease prognosis scoring and

response to treatment within MDS and MPN, describe the current advances made in characterising the role of *CALR* and *CD47* in solid tumours and the emerging evidence for a role of *CALR* and *CD47* with in MDS and MPN. We will also evaluate how these advances in our understanding of the role of *CALR* and *CD47* are starting to aid as diagnostic markers and in therapeutic treatments for these myeloid malignancies.

Challenges with Characterization and Prognostic Scoring within Myelodysplastic Syndrome and Myeloproliferative Neoplasms

Whilst both MDS and MPN are haematopoietic stem cell disorders that tend to develop on to AML; there are distinct differences between these disease subtypes. MDS develops from a group of clonal haematopoietic stem cell disorders, which are characterised by ineffective haematopoiesis which can develop into cytopenias [4]. Prognostic scoring of MDS is usually characterized on the basis of the extent of cytopenia, the percentage infiltration of the bone marrow blasts, and abnormal chromosomal structure upon karyotyping [5]. MPNs are chronic myeloid cancers that are characterized by over production of mature blood cells, which have the potential to evolve into AML [6]. The three most common forms of MPN are Essential thrombocythemia (ET), polycythaemia vera (PV) and myelofibrosis (MF) [7], which are generally well defined from each other but quite often with overlapping presentations. Somatic mutations are common in MPNs; in the majority of patients *JAK2* mutations, which affect *JAK/STAT* pathway signalling, have been described [8]. Although other mutations in MPN have been acknowledged, such as the *CALR* mutations or *cMPL* mutations [9], it is thought that all roads lead to

JAK/STAT pathway, with these other mutations also affecting downstream parts of the JAK/STAT pathway.

Prognosis of MDS and MPN heavily depends on type and severity of disease. Over the past 25 years classification systems (French-American-British [FAB] and World Health Organization [WHO]) along with several prognostic-scoring systems, such as the International Prognostic Scoring System (IPSS), have been widely used [7,10,11]. Vast heterogeneity in these disorders results in a wide range of clinical indicators including: signs and symptoms of anaemia, infection and bleeding complications predominate, with systemic symptoms or features of autoimmunity being seen in some patients; perhaps indicative of disease pathogenesis [4].

In MDS, a standard measuring system ranking the risk factors of each disease into: very low, low, intermediate, high and very high risk is currently used (IPSS-R) [11] while in MPN a similar prognostic index (Dynamic International Prognostic Scoring System [DIPSS]) is only informative in the MF subtype, which is used for estimating survival from any point in the disease progression [12]. These scoring systems are mainly based on bone marrow/peripheral blood features, cytogenetics and some clinical manifestations such as systemic symptoms or splenomegaly but no specific genetic marker highlights specific subtypes or stages of those conditions or predicts transformation and disease progression.

Recent evidence has suggested that certain markers involved in controlling phagocytosis could be key in both disease progression and response to treatment within both solid cancer and myeloid malignancies, with the role of the pro-phagocytic *CALR* and anti-phagocytic *CD47* recently coming to prominence. Overexpression of *CALR* has been documented to produce a pro-phagocytic signal in solid tumours [13] and it is often counteracted by a concomitant expression of the anti-phagocytic *CD47* [14], reflecting an apoptosis vs survival mechanism in response to chemotherapy. This expression pattern could be used as a potential target for therapeutics and prognostic scoring, as *CALR* and *CD47*'s role in cancers, with a potential role for *CALR* and *CD47* also emerging in MDS/MPN which will be discussed further.

The Roles of Calreticulin and CD47 in Ordaining/Controlling Phagocytosis

CALR is an endoplasmic reticulum (ER) protein, which has a crystal structure comprise of an extended proline-rich P-domain inserted between N- and C-terminal domains. *CALR* is involved in many different functions such as calcium homeostasis, protein folding and post translational modifications [15]. Although *CALR* is an ER protein, it is also found throughout the cell and outside the ER is predominantly located in the membrane and the cytosol [14] where it is involved in gene expression, cell death, programmed cell removal and wound healing [16].

The structure of *CALR* is split into 3 different domains each with a specific function. The acidic C-domain is responsible for calcium regulation and binding, predominantly within the ER, and the globular N-domain and extended P-domain are primarily responsible for the protein's chaperone function [14,15]. The C-domain contains the KDEL amino acid sequence which prevents *CALR* from being secreted from the ER. Whilst the KDEL signal resides it to the ER it can however be translocated to other parts of the cell when needed [17]. KDEL-containing proteins bind to KDEL receptors on the ER affecting its translocation. If the protein is missing or has a mutated KDEL

region cellular mis-localisation can occur. Localisation studies show that in myeloid cells *CALR* is upregulated, mutated, *CALR* stays located to the ER [17].

Expression of *CD47* affects cellular proliferation, apoptosis, adhesion, migration and immune system homeostasis [18]. *CD47* is comprised of an extracellular immunoglobulin (Ig) variable domain, a five times transmembrane-spanning domain, and a short alternately spliced cytoplasmic tail [18]. Different *CD47* tissue expression patterns can be seen, which has been proposed to be due to different isoforms of *CD47* determined by alterations in the range of length of the cytoplasmic tail from between 4-36 amino acid long [19].

The role of *CD47* is in vacillating and increasing IgG mediated phagocytosis when responding to the presence of the arginine-glycine-aspartate (RGD)-containing extracellular matrix proteins [18]. As a cell surface glycoprotein of the Ig superfamily, *CD47* is a transmembrane protein that interacts with several molecules including the protein signal regulatory protein alpha (*SIRPα*) [19,20]. *CD47* blocks phagocytosis mediated by the *SIRPα* protein. Binding to *SIRPα*, *CD47* stimulates the *SIRPα* to initiate a signalling cascade. Signalling causes recruitment of the src homology-2 domain containing protein tyrosine phosphatases SHP-1 and SHP-2, resulting in signalling to inhibit phagocytosis [21], giving *CD47* the title of the "don't eat me signal" [22].

CALR and *CD47* in Cancer Prognosis

CALR expression on the cell surface acts as the pro-phagocytic signal activating the clearance of aged or damaged cells [23], but also, as described in different human cancers (ie AML, acute lymphocytic leukaemia, chronic myeloid leukaemia, ovarian cancer, glioblastoma, and bladder cancer [13] has roles in calcium signalling, macrophage-mediated immune evasion and activation of the unfolded protein response (UPR), which is responsible for upregulation of molecular chaperones and degrading misfolded proteins. In solid tumours this is often counterbalanced by the concomitant expression of the anti-phagocytic *CD47* [14]. Overexpression of *CALR* mRNA and cell surface over-exposure of *CALR* correlates with poor prognosis in mantle cell lymphoma, superficial and invasive bladder cancer, and neuroblastoma [13]. Chao et al. [13], showed a correlation between *CALR* expression and survival with high expression of *CALR* associating with worse survival and high counteracting over expression of *CD47*.

In some MPN cases, mutation within exon 9 of *CALR* has been found, causing loss of the C-terminal acidic domain, multiple calcium-binding sites, and the KDEL sequence [24] resulting in a dysfunctional protein and contributing to over-activation of JAK/STAT pathway leading to myeloproliferation [17]. However, mutated *CALR* has also been shown to cause abnormal localising expressions in the cytosol and membrane [24,25] and activation of the UPR, which is often linked to disease progression *via* arresting protein translation through the ER compartment [26].

Several studies have shown that targeting *CALR* translocation to the cell membrane may represent a new strategy in cancer therapy [27,28]. Translocation from within the cell to the cell membrane is required for the initiation of the pro-apoptotic signalling of *CALR* [15]. Treatment with chemotherapy drugs anthracyclines and the reticulum Ca^{2+} ATPase Inhibitor thapsigargin, induces *CALR* translocation to the cell surface *via* Endoplasmic Reticulum Resident Protein 57 (ERp57) (a member of disulfide isomerase family), enhancing the immune

response and resulting in apoptosis of the cancer cell [27,29]. In contrast, knocking out causes a decreased surface expression of *CALR* and prevents the cells from being attacked by T-cells resulting in a cell survival benefit [30].

Expression changes in *CD47* are a marker of phagocytosis, expression in aged or damaged cells lead to homeostatic phagocytosis. Patients suffering with cancer show high expression rates of *CD47* which correlate clinical outcomes. This is shown in both solid tumours [21] and also in haematological cancers including AML [31], when *CD47* expression increases on the surface of cancer cells it signals for survival by evading immune surveillance, acting as a marker of anti-phagocytosis [32,33].

Emerging Role for *CALR* and *CD47* in Myeloid Disorders

The *CALR/CD47* expression ratio seems to direct the immune response in MDS/MPN in different ways depending on which signal is predominant, with high *CALR* expression signalling for phagocytosis and high *CD47* expression protecting the cancer cells from attack by the immune system [34]. Macrophage-mediated immunosurveillance is a form of programmed cell removal (PrCR) by the immune system, where target cells are recognized and phagocytosed [35], which is independent of apoptotic removal of cells by the immune system. Studies have shown *CD47* to be the protector of cancer cells from the PrCR by overpowering the PrCR signals [34]. Blocking *SIRPα* and *CD47* allows for PrCR of cancer cells and not normal cells to be targeted due to the higher expression of the eat-me signals, such as *CALR*, which are commonly expressed in cancers in cell lines, animal models and patients [34]. The binding of *CALR* on the surface of a cell to LDL receptor related protein 1 (LRP1) receptor on macrophages is a vital mechanism to induce phagocytosis in targeted cells [13]. Interestingly, upregulation of *CALR* on the surface of cancer cells is usually followed by a concomitant upregulation in cell surface expression of the anti-phagocytic *CD47*, in a survival response to keep the cell alive and evade immunosurveillance [34]. Cells being able to evade immunosurveillance is a key hallmark for cancer [36].

Clearance of the tumour cells can also be triggered by blocking interaction between *SIRPα* and *CD47*, allowing for *CALR* phagocytosis signalling on the cell surface to become predominant [37]. MPN exhibits mutations in *CALR* (altering its function), seen in 67% of patients with ET and 88% of those with MF who do not have a *JAK2* or *MPL* mutation [38]. It has been shown that only mutated *CALR* cells resulted in phosphorylation of *STAT5* which subsequently activates the *JAK/SAT* pathway [39], indicating *CALR* to be directly involved into MPN pathogenesis and cells proliferation [40].

Role of *CD47* expression in MPN and MDS has not yet been well defined and characterized, however, membrane over-expression of *CD47* has been measured in AML [31,41] suggesting *CD47* as a potential target in AML therapy. It is known that patients with MDS, can progress on to AML throughout the course of their treatment. A gradual increase in *CD47* expression has been demonstrated in MDS cells progressing to AML with lower risk patients expressing the lowest level comparing with higher levels in high risk MDS. Jiang et al. [41] showed that patients with high risk MDS overexpressed *CD47*, but exhibited lower levels of *CALR*, while in low risk patients it was the opposite suggesting a potential role *CD47/CALR* ratio in disease progression and prognosis [13]. Interestingly, in post MDS-AML patients the level of expression of *CALR* is significantly lower than in

de novo AML (MDS-AML: 10%; *de novo* AML: 25%) suggesting a different immune response in secondary AML and potentially a clonal evolution dictated by immune selection. Loss of *CALR* is detrimental to cell phagocytosis, allowing the anti-phagocytic signal *CD47* to evade phagocytosis resulting in a poor prognosis [13]. The role of *CALR* and *CD47* in MPN is less clear. Post MPN AML cell lines and patients do not seem to overexpress *CD47* as post MDS AML cell lines do suggesting a possible different mechanism of immunoresistance, however preliminary data of our laboratory, suggest an upregulation of *CD47* but not *CALR* in chronic phase MPN (unpublished observation).

CALR and *CD47* targeting for Anti-cancer and Myeloid Malignancy Therapy

CALR can be used as a potential target for anti-cancer therapy using molecules able to enhance *CALR* expression and stimulate phagocytosis of the affected cells [15]. In prostate and lung cancer cells, *CALR* targeting adenovirus (Ad-*CALR/MAGE-A3*) have produced an up regulation of *CALR* and consequently an increased rate of apoptosis in the cancer cells [42]. Administration of a chimeric construct of *CALR* along with a chemotherapy agent increased treatment response in mice with mammary tumours [43], indicating the potential combined therapy in mammary tumours. In addition, after treatment with anthracycline, *CALR* expression on the cell surface correlated with the overall removal of cancer cells and survival of the patients [44], resulting in the activation of a tumour-targeting immune response in AML, neuroblastoma, non-small cell lung carcinoma, ovarian cancer, and colorectal carcinoma [44].

Anti-*CD47* antibodies can block *CD47-SIRPα* signalling and induce cancer cells death. Dheilly et al. [45] showed how combining an anti-*CD47* with an anti-*CD49* was effective in inducing cell death in leukemic, lymphoma, pancreatic, and ovarian carcinomas cell lines. Chemotherapy drugs are considered as the first line treatment for cancers, however, chemotherapy is not always effective. Combination therapies have been proposed, using specific chemotherapy drugs in conjunction with anti-*CD47* [46]. Liu et al. [47] generated pre-clinical assessment of a humanised anti-*CD47* antibody (Hu5F9-G4) which in combination with rituximab chemotherapy drug treatment, resulted in an initial reduction of all blasts *in vivo*. After day 159 blood counts (white blood cells, hemoglobin, and platelets), were seen to return to normal range [48]. Concomitantly, Wang et al. showed that combining cytarabine in conjunction with anti-*CD47* antibody, increases the antitumor effects in AML comparing with cytarabine alone [48].

Blocking *CD47* using *CD47 siRNA* could also represent a therapeutic strategy. Wang et al. [49] showed efficacy of *CD47 siRNAs* in melanoma and lung metastases regression. The *CD47 siRNA* were intravenously administered and resulted in reduction of tumour size. Gholamin et al. [50] demonstrated inhibition of *CD47* using humanized anti-*CD47* antibody Hu5F9-G4, which increases the presence of activated macrophages in brain tumours. *SIRPα* dependent phagocytosis induced this by blocking the *CD47* signal [31]. Macrophages then become active due to the increase in the phagocytic signals, activating macrophages to phagocytose cancer cells [34]. Further studies are being carried out into both *CALR* and *CD47* targeting which makes the future of the area very interesting.

Conclusion

The role of *CALR* and *CD47* in myeloid neoplasms is currently poorly understood. In solid tumours overexpression of *CALR* produces a pro-phagocytic signal and it is often counteracted by a concomitant expression of the anti-phagocytic *CD47*. Over expression of *CALR* has been seen to correlate with poor prognosis as it is counteracted by increased *CD47* expression, which is critical for cancer cell survival. Several studies demonstrated how *CD47* is often overexpressed in AML, allowing the leukaemia cell to evade immune surveillance and survive. During MDS progression onto AML, the levels of expression of *CALR* increase but is immediately counteracted by the concomitant *CD47* overexpression, resulting in a predominate anti-phagocytic message. The expression of *CD47* in MPN is not well defined yet. Preliminary data from our laboratory indicate in MPN samples no increase of *CALR* expression is seen on the cell surface, whilst *CD47* expression is significantly elevated in ET, PV and MF, indicating a possible different mechanism of immunoresistance comparing to solid tumour and AML.

Emerging therapeutic strategies targeting *CALR* and *CD47* expression have offered an interesting insight into the roles of *CALR* and *CD47* in myeloid neoplasms and their implication into pathogenesis and disease progression. Targeting *CALR* with the aim of increasing its expression on the cell surface using an adenovirus (Ad-*CALR*/MAGE-A3) resulted in significant increase in cell death and apoptosis. Blocking the *CD47*-SIRP α signalling pathway by using Hu5F9-G4 has resulted in reduction of *CD47* on cancer cells and consequently increases in cancer cell removal and tumour burden reduction.

CALR and *CD47* play vital roles in the pro/anti - phagocytic mechanisms of cancer cells. Better understanding is required for their involvement in myeloid malignancies. *CALR* and *CD47* targeted therapies alone or in combination with standard chemotherapy could result in better outcome for patients suffering with both haematological and solid cancers. Further studies are required to better fully understand the implication of *CALR* and *CD47* expression/regulation and their prognostic role in disease progression and treatment response in cancer and the myeloid malignancies.

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