Chapter

Membrane-Bound Complement Regulatory Proteins in Breast Cancer: Are they Best Therapeutic Targets?

Sofia Álvarez-Lorenzo, Rebeca Elizabeth Montalvo-Castro, Jeannie Jiménez-López, María Adriana Medina-Mondragón and Nohemí Salinas-Jazmín

Abstract

Breast cancer is one of the most aggressive diseases in women, responsible for thousands of deaths annually and millions of new diagnoses; its treatment presents multiple obstacles due to late diagnosis and the various mechanisms of tumor resistance. In breast cancer the membrane-bound complement regulatory proteins (mCRP) have been proposed as biomarkers of malignant cellular transformation. These are molecules capable of inhibiting therapeutic efficacy, from both antibodies and cytotoxic drugs. Therefore, these proteins are potential targets to increase therapeutic efficacy and avoid cancer progression. We will gather information about mCRP: (i) structural features; (ii) expression levels in breast cancer and relationship with prognosis; (iii) therapeutic resistance mechanisms; and (iv) strategies to down-regulate mCRP in both activity and expression.

Keywords: breast cancer, mCRP, therapeutic resistance, therapeutic mAb, systemic treatments

1. Introduction

Cancer is one of the most fatal diseases in the world, and breast cancer is the most incidence and mortality in women [1]. Breast cancer is a highly heterogeneous disease with morphological features and variable clinical outcomes. The clinical course, prognosis, and responsiveness to breast cancer treatment depend on their specific biological characteristics or classification. The immunohistochemical classification is based on hormone receptor (HR) expression (estrogen receptor [ER] and progesterone receptor [PGR]) and amplification of the human epidermal growth factor receptor ERBB2/HER2-: the HR-positive (luminal A or B), the HER2-positive and triple-negative (TNBC) subtypes [2, 3].

Overall, the systemic therapy administered consists of endocrine therapy for all HR+ tumors, immunotherapy plus chemotherapy for all HER2-positive tumors, and

cytotoxic chemotherapy plus immunotherapy for TNBC [4–7]. It has been reported that long exposure to therapeutic agents may generate an adaptive cellular response that results in the induction of acquired drug resistance. So, the use of combination chemotherapy potentially provides advantages such as chances for increasing or maintaining efficacy and reduced or delayed development of drug resistance [8].

However, there are many factors involved in the failure of treatment, such as the expression of complement regulatory proteins (mCRP). These proteins have been reported to be up-regulated in several cancer cells and tumor tissues, as a mechanism to evade elimination by the complement system [9–11].

High expression of mCRP by cancer cells confers resistance against antitumoral therapies by controlling the activation of the complement cascade and regulation of intracellular complement signaling in cancer cells [11–15].

Herein, we summarize evidence related to mCRP tumoral activity in cancer cells and discuss the implications of its biological actions in anticancer therapy. Therefore, we will gather information about mCRP: (i) structural features; (ii) expression levels in breast cancer and relationship with prognosis; (iii) therapeutic resistance mechanisms; and (iv) several strategies to down-regulate mCRP in both activity and expression.

2. The complement system and breast cancer

2.1 Breast cancer treatment

Breast cancer is the most frequently diagnosed cancer in women comprising 24.2% of total cancers, and is the leading cause of cancer mortality in women worldwide (15.5%), constituting a complex public health problem [16]. On the molecular level, breast cancer is a heterogeneous disease, and it has been classified according to gene expression patterns and the presence of specific molecular markers in tumors. The main molecular markers considered are the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (ERBB/HER2) because of their relevance in cancer pathogenesis and their prognostic value in treatment response. In clinical practice, the detection of these markers by immunohistochemistry (IHC) allows tumor classification. Tumors expressing ER and PR are considered hormone receptor-positive; those that exhibit HER2 amplification or overexpression are HER2-positive, and tumors lacking expression of ER, PR or HER2 are triple-negative [2, 4, 17].

Systemic therapy for breast cancer is determined by subtype; patients with HR-positive tumors receive endocrine therapy, and a minority also receive chemotherapy. Patients with HER2-positive tumors receive HER2-targeted monoclonal antibody (mAb) or small-molecule inhibitor therapy combined with chemotherapy, and patients with triple-negative tumors usually receive chemotherapy [4, 5]. Several antibody-based treatments targeting tumor antigens and tumor-promoting signaling pathways have been shown to rely on complement system activation for mAb-induced cytotoxicity, including the HER2-specific mAbs trastuzumab and pertuzumab (IgG1 isotype). Their Fc regions interact with the complement component C1q, inducing activation of the classical complement pathway. Moreover, the Fc regions can also interact with FcγRs of natural killer cells, macrophages, and neutrophils to induce antibody-dependent cellular cytotoxicity (ADCC). Phagocytosis of tumor cells by phagocytes is also enhanced by complement fragments such as C3b, in a mechanism

referred to as complement-dependent cell-mediated cytotoxicity (CDCC) [10]. Not only trastuzumab and pertuzumab but also other mAbs such as sacituzumab-govitecan, an anti-Trop-2 IgG1 antibody used for the treatment of triple-negative breast cancer, follow these mechanisms of action [7].

Despite their efficacy, intrinsic or acquired resistance to mAbs-based treatments occurs frequently. For example, about 70% of HER2-positive breast cancer may have intrinsic resistance to trastuzumab, and most of those who respond to this treatment tend to develop acquired resistance within 1 or 2 years [18]. Several mechanisms may lead to antibody resistance, e.g. down-regulation of the target epitope, diminished ADCC or opsonization, or resistance to complement-mediated lytic attack. There is ample evidence that complement resistance of tumor cells is a widespread phenomenon, and therefore strategies to overcome this problem are needed [19].

2.2 The complement system

The complement system is part of the innate immune response, and it represents one of the first lines of defense against pathogens. However, it also plays crucial roles in maintaining homeostasis through mechanisms such as the removal of apoptotic cells, the regulation of coagulation, angiogenesis, lipid metabolism, and importantly, the surveillance of cancer cells [9]. Complement functions through a series of over 30 coordinated cascading proteins and zymogens to induce cellular lysis, opsonize pathogens, induce inflammation and interact with cells of adaptive immunity [20].

Depending on the activator, complement can be triggered by three different pathways: classical, lectin, and alternative (**Figure 1**).

- The classical pathway is activated by the binding of C1q, in complex with C1r and C1s serine proteases, to the Fc region of immunoglobulins (IgG or IgM) complexed with antigen. The binding of C1q to a ligand results in a conformational change leading to the sequential activation of C1r and C1s. Activated C1s cleaves C4 into C4a and C4b, and C2 into C2a and C2b. Subunits C4b and C2a form C4bC2a, a C3 convertase enzyme complex able to cleave C3.

- The lectin pathway is analogous to the classical one, but its activation is triggered by mannose-binding lectins (MBLs), collectins or ficolins that bind to carbohydrate ligands such as mannose, and together with MBL-associated serine proteases (MASP1,2) form a C1-like complex, leading to the formation of a C3 convertase.

- Activation of the alternative pathway occurs through spontaneous hydrolysis of C3 to C3(H20), often referred to as "tick-over mechanism" that leads to a constitutive low level of complement activation. C3(H20) binds to factor B (FB), which is then cleaved by Factor D, and the Bb fragment forms the C3(H20) Bb complex. This fluid phase complex cleaves plasma C3, resulting in C3b, which binds to cell surfaces and Bb, generating C3Bb, the C3 convertase of the alternative pathway.

In all pathways, the C3 convertase generates a C5 convertase by binding to C3b molecules. Then, C5 convertase cleaves C5 to create C5b which binds with C6, C7, C8, and multiple C9 to form the C5b-9 complex or membrane attack complex (MAC) which functions as a pore in the cell membrane that leads to cellular lysis [9, 19–21].

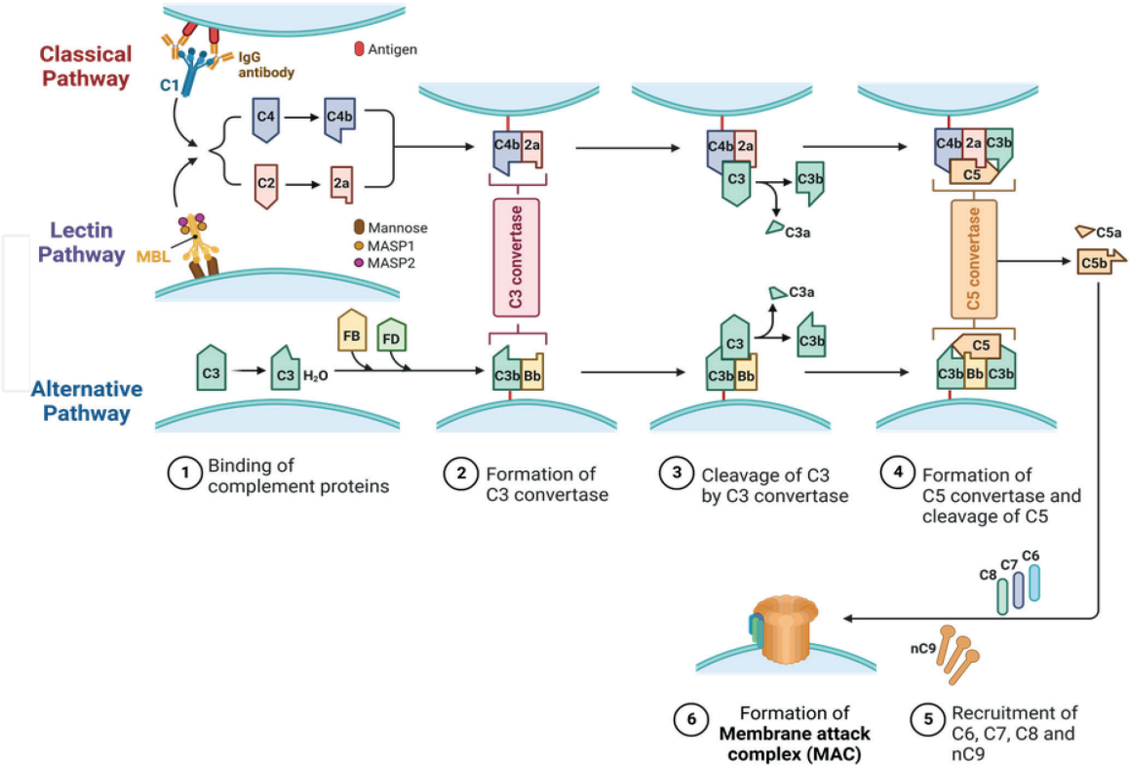

Figure 1.
The complement system activation pathways. Depending on the context, complement system can be initiated by three distinct pathways: classical, lectin, and alternative, each leading to lead to the formation of C3 and C5 convertases and the common terminal pathway, in which the formation of the membrane attack complex (MAC) leads to cellular lysis. Created with Biorender.

While inducing cell lysis through MAC is an important effector arm, the complement system can also trigger pro-inflammatory signaling and phagocytic functions that are equally important. C3a and C5a function as anaphylatoxins and are constantly released during complement activation. These molecules recruit and induce activation of immune cells expressing anaphylatoxin receptors (C3aR, C5aR1, C5aR2) such as neutrophils, monocytes, eosinophils, mast cells, and macrophages. Furthermore, C3b and C4b function as opsonins that aid in phagocytosis by binding to the target cells surface, allowing the elimination of pathogens and stressed cells [9, 21].

2.3 Complement activation by tumor cells

It has been recognized that cancer cells acquire several genetic and epigenetic abnormalities that induce the expression of tumor-associated antigens, which may target tumor cells for recognition by complement proteins. The classical pathway has been found to be activated by the recognition of post-transcriptionally modified tumor-specific antigens by natural antibodies. Natural antibodies are predominantly IgM isotype and are produced without prior antigenic stimulation against a variety of self and foreign antigens. Furthermore, IgM antibodies can effectively activate the classical complement pathway because, unlike IgG, a single molecule of IgM can bind to C1q and initiate the proteolytic cascade [9, 22]. However, the activation of the classical pathway through IgG antibodies in breast cancer is not excluded, as is the case with therapeutic antibodies [5, 12]. The presence of IgG, C3, and C4 together with deposits of C5b-9 complexes on tumor cell membranes, were observed in samples

from breast cancer patients, indicating a persistent *in situ* complement activation [23]. In addition, altered glycosylation patterns reported in breast cancer cells, such as an increased expression of α2,3-sialic acid and α-L-fucose [24], are likely to induce activation of the complement via the lectin pathway.

Although complement activation may favor the elimination of tumor cells, the role of this system appears to be more complex in the context of the tumor microenvironment (TME). Recent studies demonstrate that the impact of complement in cancer is diverse, ranging from anti-tumor defense by killing antibody-coated tumor cells, to potent tumor promotion by supporting local chronic inflammation or interfering with anti-tumor T-cell responses. Indeed, complement molecules C3, C3a, and C5a are reported to play an important role in cancer progression. For example, in mice models of breast cancer, C3 expressed by CD8+ T cells inhibits their antitumor activity through an autocrine mechanism [25], whereas C5a/C5aR signaling promotes metastasis by the recruitment of myeloid-derived suppressor cells (MDSC) in premetastatic sites, causing suppression of effector CD8+ and CD4+ T cell responses [26]. In contrast, other complement components act as tumor-inhibiting factors. For example, C1q deficient mice exhibit accelerated tumor growth and an increased number of lung metastases, which are not directly related to absence of complement activation, but to the induction of angiogenesis and an increase in HER2 expression [27]. Taken together, studies suggest that opposing effects of complement in cancer are dependent upon the sites of activation, the composition of the TME, and the tumor cell sensitivity to complement attack [28].

3. Characterization of the mCRPs: structure, localization, and function

Complement activation must be a tightly coordinated orchestra through any of its pathways to avoid damage to its tissues. Multiple negative regulators are known as complement regulatory proteins (CRP); their function is to maintain homeostasis in the system. Among them, we can mention two main groups: (i) soluble complement regulatory proteins (sCRP), such as C1 inhibitor, C4b binding protein, and factors H, B, D, and I; and (ii) membrane-bound complement regulatory proteins (mCRP), which include CD35, CD46, CD55, and CD59 [9]. Mostly CD46, CD55, and CD59 have received more attention since they are overexpressed in tumor tissues, and their complement inhibitory functions have been proposed as resistance strategies applied by cancer cells [9, 11, 29, 30].

3.1 CD46

Membrane cofactor protein (MCP) known as CD46 is a type 1 transmembrane glycoprotein, with a molecular weight that varies between 48 and 68 kDa. Was discovered on peripheral blood cells in 1986 during a search for novel C3b-binding proteins [31] and renamed as "membrane cofactor protein" due to the growing structure/function information in 1991 [32]. This protein has a structural heterogeneity, partly explained by the expression of multiple cDNA/protein isoforms that arise by alternative splicing of serine/threonine/proline-rich exons (sites of heavy O-glycosylation) and cytoplasmic tails (**Figure 2**) [33]. CD46 is expressed in all nucleated cells, thus only erythrocytes lack CD46 expression [34]. The gene for this protein is encoded on chromosome 1q32.2 and its transcription depends on binding the activated transcription factor STAT3 to its promoter [35].

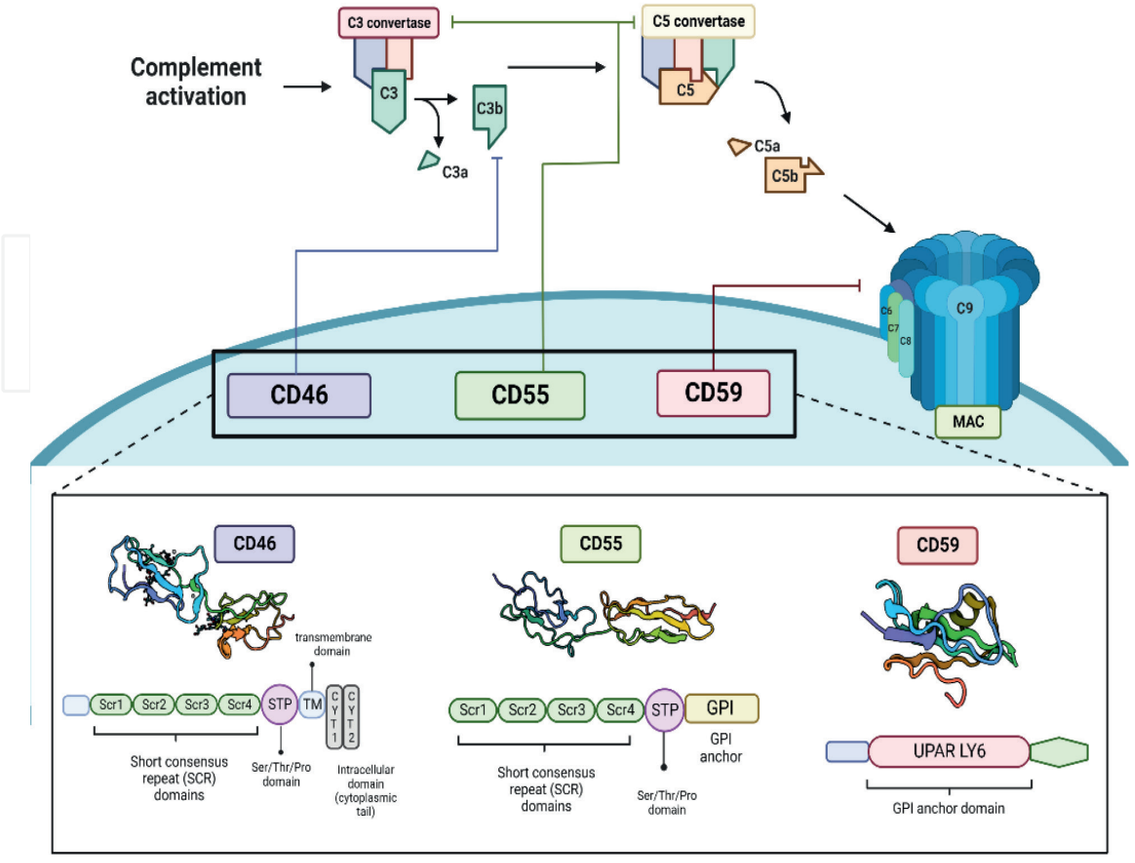

Figure 2.
Membrane-bound complement regulatory proteins: structure and function in the complement system inhibition. Created with Biorender.

CD46, belongs to the family of regulators of complement activation (RCA), elucidated since 1985. Its structure is common to all proteins expressed from the RCA family; it is mainly based on four short consensus repeat (SCR) domains that make up most of the extracellular region, the SCR repeats are connected to a hooking region rich in serine, threonine, and proline (STP region), a single membrane-spanning segment (transmembrane domain), and a cytoplasmic tail divided into two domains, identified as CYT1 and CYT2 [32, 36, 37]. CD46 inhibits the formation of complement C5 convertase by promoting the degradation of C3b and C4b by proteolytic cleavage [38, 39]. Biochemical mapping studies strongly implicate the SCR2, SCR3, and SCR4 domains in the interaction of this protein with complement cascade proteins (**Figure 2**) [40, 41].

CD46 can be proteolytically modified on cell membranes and released by a metalloproteinase from cancer cells as vesicles with a diameter of 200 nm. Both vesicular and soluble forms of CD46 are functional and promote C3b cleavage by factor I [42].

3.2 CD55

CD55, also known as Decay-accelerating factor (DAF), was first discovered in 1969 on the cell surface of an erythrocyte. It is a glycophosphatidylinositol (GPI)-anchored cell surface glycoprotein with a molecular weight that varies from between 50 and 100 kDa depending on cell type [43]. DAF is present in most cell types; its gene is encoded on chromosome 1q32.2 located adjacent to the genes comprising the RCA gene family, which includes as well CD46. Its expression is primarily modulated at

the transcriptional level by a cAMP response element on its promoter and by the Sp1 transcription factor. The most abundant variant generated by alternative splicing is found in the membrane [44].

The main function of CD55 is to protect cells from autologous complement attacks. Its role as a complement regulator is accelerating the dissociation and disintegration of C3 convertase and preventing the formation of C5 convertase, avoiding cell damage due to the subsequent formation of the MAC [45, 46]. Mature CD55 has three domains: (i) consensus repeat domains consist of four short consensus repeat (SCR) domains e.g. SCR-1, SCR-2, SCR-3, and SCR-4; (ii) O-linked carbohydrate domain having a serine/threonine/proline-rich region, and (iii) GPI anchor [47]. The final GPI-anchored domain of CD55 binds the protein to the membrane's outer leaflet at dynamic structures composed of sphingolipid and cholesterol, called lipid raft microdomains (**Figure 2**). This segment is composed of about 30 amino acids cleaved post-translationally at the C-terminal signal peptide located at Ser352 during processing at the endoplasmic reticulum (ER) and its later modification in the Golgi apparatus before transporting to the cell membrane [48].

Has been reported that cell-surface CD55 is a ligand for CD97, a member of the epidermal growth factor seven-span transmembrane (EGF-7TM) receptor family; the binding of CD55 to CD97 can protect several cell types from complement-mediated damage, thus playing an important role in host defense and inflammation [43, 49]. Furthermore, CD55 can stimulate CD97 signaling and modulate cancer metastasis, as a mechanism dependent on the upregulation of MMP2 and MMP9 [50].

The CD55 signaling can be activated by growth factors, cytokines, and augment prostaglandins [43]. In Hep3B hepatoma cells, the exposition of cytokines such as TNF-α, IL-6, and IL-1β increased the expression of CD55 (three-fold) and CD59 (two-fold) and decreased the expression of CD46, demonstrating the relevance function of TME and inflammatory cytokines on the expression of mCRP [51]. Another example of the fine regulation of CD55 expression in HT-29 colon cancer cells depends on the activation via p42/44 MAPK pathway by the epidermal growth factor (EGF) [52].

3.3 CD59

CD59 or membrane attack complex inhibitor (MAC-i), is a glycoprotein of 20 kDa encoded on chromosome 11p13, expressed in most cells. It belongs to a protein superfamily characterized by the expression of a Ly-6/uPAR domain (**Figure 2**), which allows it to interact with complement proteins [53, 54]. This protein was first described in 1986 and multiple groups worked on its characterization; in 1988 was identified in human lymphoid cells and designed as a "membrane attack complex inhibiting protein" [45, 55]. Their role in the regulation of the complement system consists in inhibiting cell lysis by binding to the α chain of C8 and the β domain of C9 to prevent MAC formation in the membrane of cells, including cancer cells [56].

CD59 has three folded β sheets and one α helix; it has a cysteine-rich Ly6/uPAR three-finger domain, a characteristic pattern of disulfide bonds, and a unique group of amino acids susceptible to N- and O-glycosylation (Leu1-Asn77) that constitute the core of the molecule. It has been proposed that these glycosylations may influence its membrane distribution, limiting the spatial orientation of the extracellular domain to interact with membrane attack complex (MAC) proteins and preventing their digestion by proteases [54]. This protein requires the presence of detergent-insoluble glycolipid rafts (DGI) and the glycosylphosphatidylinositol (GPI) anchor to remain in the cell membrane [53]. Its membrane binding with GPI allows it to activate

intracellular signaling to promote carcinogenesis, enhance cell adhesion, migration and signaling through binding to vitronectin and interactions with integrins in breast cancer cells [57].

Its expression takes place under different contexts: (i) constitutively regulated by the Sp1 transcription factor, (ii) induced under inflammatory conditions by scaffolds between NF-κB and CREB proteins bound to CBP/p300 [58], (iii) conditionally regulated by the transcription factor Smad3 induced by TGF-β during the epithelial-mesenchymal transition (EMT) [44], and (iv) selectively expressed by SOX2 in populations of cancer stem cells (CSC) [59].

4. mCRP expression in breast cancer and prognosis in patients

Expression of CD46, CD55, and CD59 in breast tumor samples, through PCR analysis, showed that CD46 is the most highly expressed mCRP in breast cancer [60]. In addition, IHC analysis confirmed the high expression of CD46 with both cytoplasmic and membrane staining; however, there are controversies regarding the expression of CD46 and its impact on prognosis [61, 62]. In samples of patients with primary invasive tumors, CD46 was highly expressed in most samples (99.4%), and the intensity of its expression has an inverse correlation with tumor grade histological, and tumor size. Furthermore, intense staining of CD46 was found in good prognosis-type tumors (tubulo-lobular, tubular, mucinous, and invasive cribriform types). In contrast, it was less common in poor prognosis types (ductal/NST, solid lobular, lobular mixed, mixed NST, and lobular types). Finally, older patients had a higher expression of CD46 [61]. However, another study showed that patients with CD46 negative tumor have a better prognosis, with an increased progression-free time and overall survival time compared to patients expressing CD46. All patients in this study underwent post-operative radiotherapy, while the patients in the first study did not. This could explain the different results between the two studies [62].

It has also been reported that ER-positive tumors overexpress CD46 and that its expression confers a loss of differentiation of tumors, a characteristic strongly related to aggressiveness. This is consistent with the report of Thorsteinsson et al. on the upregulation of CD46 during malignant progression [60, 63].

CD55 is also highly expressed in breast cancer: in a study with 74 samples, 50 of them (67.6%) were categorized as CD55-high and the remaining as CD55-low, with a strong positivity in stage II and III tumors. Immunohistochemistry analyses also showed a high expression of CD55 in the cytoplasm of the cells [64]. Furthermore, a strong correlation exists between patients with CD55-high tumors and a shorter relapse rate. Thus, CD55 could be a recurrence prognostic factor [64]. Madjd et al. report that after therapy, surviving cells may overexpress CD55 on breast tumors as a response to complement activation by the tumor environment [61]. On the other hand, a study of 480 cases of primary operable invasive breast carcinoma reported a higher expression of CD55 in grade 1 or 2 tumors, and this high CD55 expression correlates with a good prognosis [65]. This same study revealed that the loss of CD55 might also correlate with poor prognosis, which is consistent with another report that establishes that during malignant progression, there appears to be a downregulation of CD55. This could be due to the protein's role in regulating the immune response via interaction with its ligand, CD97 [63, 65].

An analysis of clinical specimens from 120 patients (58 with lung metastases and 62 without metastatic disease) revealed that patients with high CD59 expression

mCRP	Expression	Samples	Treatment	Prognosis
CD46	High	70	Chemotherapy + radiotherapy	Unfavorable [62]
		510	Chemotherapy	Good [61]
CD55	High	74	Only surgery	Poor: relapse [64]
		480	Radiotherapy +/− chemotherapy	Good [65]
CD59	High	120	—	Poor [66]
		520	No information	Good [67]

IHC: immunohistochemistry.

Table 1.
Comparison between different reports on mCRP expression and impact on prognosis in breast tumor samples evaluated by IHC.

might have a worse prognosis, as there was a positive association between CD59 expression and metastasis. Therefore, patients with high CD59 expression are more likely to develop metastases [66]. Kaplan-Meier analysis showed that the expression of CD59 in breast cancer patients correlates with a worse relapse-free survival rate and, furthermore, it appears that CD59 upregulation occurs during malignant progression. Thus, CD59 may be a prognostic biomarker of poor outcomes in patients with breast cancer [63, 66]. However, as with CD55, it has been reported that the loss of CD59 could be correlated with poor prognosis due to its role in regulating the immune response via interaction with its ligand, CD2. In addition, the same study revealed that high levels of CD55 are associated with a good prognosis of moderately differentiated tumors [67]. In contrast, another study revealed that breast cancer patients with CD55 or CD59 overexpression had a higher relapse rate than those with low CD55 expression. Similarly, the mean disease-free survival of patients with CD55 or CD59 overexpression was significantly shorter than that of patients with low CD55 expression. Multivariate analysis confirmed that CD55, but not CD59, was an independent risk factor of recurrence [68].

Due to conflicting results between different studies, it is essential to consider the differences between them: the sample size, the treatment prior analysis, and scoring criteria (**Table 1**). For instance, in some studies, to categorize each case as CD55-high or CD55-low, the grade of CD55 staining intensity in each tumor cell was multiplied by the proportion of CD55-positive cells among the total tumor cells. In contrast, other studies classified as high-CD55, those cases with >1% of tumor cells showing strong CD55 expression. Because only tumor cells with significantly strong CD55 staining were counted, the proportion of CD55-low cases was higher than in the previous study [64]. Another important factor is the subtypes of breast tumors, described previously, which have different characteristics that could affect either mCRP expression or its correlation with prognosis, and none of the studies defined the type of the samples they analyzed. This could also explain the differences in the results between studies.

5. mCRP and resistance mechanisms

For many decades, antitumor therapies have improved; however, despite significant progress in cancer therapy clinical oncologists often face a major impediment to

anticancer drug resistance: intrinsic resistance from the start of therapy or after initial responses and in repeated courses of drug treatment, acquired resistance. Here, our contribution to understanding the underlying molecular basis of the role of mCRP in therapeutic resistance.

Some conditions might affect the expression levels of mCRP and, therefore, impact on the prognosis of patients. Some reports on how mCRP expression changes after chemotherapy and its relationship with resistance. Evidence indicates that mCRPs are involved in resistance to different therapeutic schemes in the treatment of patients with breast cancer. The associated mechanisms are still being studied, but it has been recognized that complement resistance conferred by mCRPs facilitates cell proliferation, survival of circulating metastatic tumor cells, poor immune response, and reduced efficacy of immunotherapy/chemotherapy.

5.1 Immunotherapy

Immune escape mechanisms limit the susceptibility of tumor cells to antibody-based therapy. The optimal efficacy of anticancer antibodies is limited by the resistance of tumor cells to complement-mediated attack, mainly through the overexpression of mCRPs [69]. Has been reported that 50% of women affected with HER2-positive breast cancer present or acquire resistance to trastuzumab.

Following the evidence that HER2-positive patients who did not respond to trastuzumab had elevated CD55 expression, CD55 and CD59 have been reported to be involved in resistance to trastuzumab or pertuzumab. Mechanistically, HER2 antibodies (trastuzumab or pertuzumab) contain IgG1 Fc that induces CDC in cancer cells by activating the classical complement pathway, thus canonical CD55 and CD59 signaling allow blockade of HER2 antibody-mediated complement regulation [13, 70]. This was studied in breast cancer cell lines by blocking CD55/CD59 activity using mAbs, modulating their expression via phosphatidylinositol-specific phospholipase C (PI-PLC) and silencing their expression using short hairpin RNA (shRNA). Results of trypan blue exclusion assays demonstrated that treatment of cells with trastuzumab incubated with pooled normal human serum (NHS) used as the source of complement, significantly enhanced CDC-dependent lysis of SK-BR3 and BT-474 cells in these three scenarios where the participation of the mCRP was inhibited [13, 68].

Another study evaluated the efficacy of trastuzumab or pertuzumab alone or in combination to induce C3 tumor cell opsonization in SK-BR3 and BT-474 cells. Enhanced deposition of activated C3 (C3d, used as a surrogate marker for the opsonization of C3b and iC3b molecules) was observed when tumor cells were incubated with trastuzumab and pertuzumab, accompanied by silencing of CD55 and CD46, but not CD59. Nevertheless, knockdown of all three mCRPs results in an optimal C3d deposition, enhanced CDC effect due to treatment with trastuzumab and pertuzumab with an overall cell lysis of 48 ± 11% in BT474 cells and 46 ± 6% in SK-BR-3 cells, as well as an increase in complement-dependent macrophage-mediated cytotoxicity of BT474 cells, analyzed by 51Cr release assay, in the presence of both trastuzumab and pertuzumab with C8 depleted human serum to avoid MAC formation [71]. These studies describe the mechanisms of resistance to immunotherapeutic antibodies due to the expression of mCRP in cancer cells. Mechanisms of resistance to Trastuzumab-emtansine, Trastuzumab-deruxtecan, and sacituzumab-govitecan due to mCRP expression have not yet been reported due to their recent approval.

5.2 Chemotherapy

After chemotherapy treatment, response rates range from 30 to 70%, but responses are often not durable as patients develop resistance [72, 73].

CD55 can also promote chemoresistance by suppressing antitumor immunity in response to neoadjuvant chemotherapy. A significantly increased tumor-infiltrating ICOSL+ B population was identified after neoadjuvant chemotherapy in samples from breast cancer patients. It has been described that tumor cell death induced by chemotherapy activates the complement system via the alternative pathway through phosphatidylserine (PS) and is responsible for inducing ICOSL+ B cells via complement receptor type 2 (CR2) [70]. CR2 recognizes complement C3 cleavage products (C3b, iC3b, and C3c) bound to antigens and acts with the B cell antigen receptor (BCR) to lower the activation threshold and overcome B cell anergy [74]. This ICOSL+CR2high B cell population can improve antitumor immune response by increasing the frequency of CD8+ T cells and Th1 cells expressing granzyme-B or perforin and decreasing Tregs in tumors. Moreover, it was identified that CD55 determines the opposite roles of B cells in chemotherapy. The role of this complement inhibitory protein in chemosensitivity and tumor immunity was evaluated in mice injected with E0771 cells (a Luminal B cell line) with or without CD55 overexpression. The results showed that doxorubicin increased tumor-infiltrating ICOSL+ B cells and effector T cells in mice bearing parental E0771 cells, but not in those bearing CD55-overexpressing cells. On the other hand, chemotherapy-induced complement C3 cleavage products analyzed by western blotting were reduced in CD55-overexpressing tumors treated with doxorubicin. Additionally, the effect of CD55 overexpression on chemosensitivity and T cell response was completely suppressed in C3−/− mice, suggesting that it is complement dependent. This evidence indicates that CD55 overexpression on breast cancer tumor cells decreases the efficacy of chemotherapy by inhibiting the induction of complement-dependent ICOSL+ B cells [75].

5.3 Endocrine therapy

Most ER+ breast cancer may initially respond to endocrine therapy, but 15–20% of tumors are intrinsically resistant to treatment, and another 30–40% acquire resistance [76].

Some conditions might affect the expression levels of mCRP and, therefore, have an impact on the prognosis of patients. There are some reports on how mCRP expression changes after chemotherapy and its relationship with resistance. On breast cancer cell lines (SK-Br3 and BT-474) it was found that tamoxifen inhibited both the protein and mRNA expression levels of CD55, potentiating the effect of trastuzumab, suggesting the combined use of trastuzumab and tamoxifen for HER2-positive breast cancer treatment [68].

A higher CD59 expression in tamoxifen-resistant breast cancer cells has been reported, both in protein and mRNA levels, suggesting that this mCRP may play a key role in tamoxifen resistance in MCF-7 cells, a luminal A cell line. Moreover, after RNAi-mediated attenuation of CD59, cells were able to overcome tamoxifen resistance, and CD59 silencing suppressed cell proliferation, indicating that CD59 plays an important role in the response of cells to tamoxifen: knockdown of CD59-induced apoptosis through changes in apoptosis-related genes: the active form of some cell programmed death factors (cleaved-caspase-8, cleaved-caspase-6, cleaved-caspase-3, cleaved-PARP proteins, and Bax/Bcl2 ratio) was increased in the CD59-silenced

tamoxifen-resistant cells [15]. These findings evidence the participation of CD59 in the regulation of pro/antiapoptotic proteins and cell proliferation in response to tamoxifen treatment to promote drug resistance in breast cancer cells.

Evidence correlating the expression of these molecules and the prognosis of patients suggests that we still have much to understand about their functions, structure, and signaling in tumor cells. As a biomarker or therapeutic target, mCRP offers several pathways for cancer therapeutics.

5.4 mCRP-expression dependent resistance but drug non-related

5.4.1 Cancer stem cells related

It has also been suggested that the overexpression of mCRPs is associated with the presence of cancer stem cells (CSC), which indicate drug resistance. The function and differentiation state of CSC are substantially modulated by many interconnected signaling pathways including IL-6/JAK2/STAT3, Hedgehog, WNT, and Notch signaling. CSCs are considered resistant to apoptosis, can modulate survival pathways and have high cell plasticity; therefore, they could survive antitumoral therapy [77].

Xu et al. reported the use of CD55 as a biomarker for CSC, after determinate high level of CD55 on mammary carcinomas (MDA-MB-231 and MCF7), two colorectal carcinomas (Lovo, RCM-1), and one lung carcinoma cell line (A549). Sorting of the CD55-high population, identified as side-population (SP) cells, revealed that cells had *in vitro* colony formation, a high self-renewal potential, and were more resistant to apoptosis in two conditions: serum depletion and ceramide addition, such as what happens with cancer stem cells. They found that anti-apoptotic proteins such as Bcl-2 were overexpressed in both SP cells and $CD55^{high}$ cells, which explain why they are tolerant to apoptosis. Researchers validated the use of high CD55 expression as a surrogate marker for sorting SP cells, which function as an identifier for CSC and, consequently, as an indicator of poor therapeutic prognosis due to the intrinsic malignancy characteristics of CSC [78]. Therefore, the authors concluded that CD55 could be an important target for CSCs, although more studies, such as the evaluation of tumorigenicity are needed.

Chen et al., reported that CD59, but not other mCRPs, was upregulated to protect sphere-forming CSCs from complement-dependent cytotoxicity. Cetuximab, a chimeric monoclonal IgG1 antibody whose specific target is the epidermal growth factor receptor (EGFR), together with normal human serum (NHS) were used to test the resistance to CDC mediated by CD59 upregulation in CSCs of MCF-7 and Calu-3 cell lines. The LDH release assay results suggested that the cell death rate was conversely correlated with the expression level of CD59. Additionally, SOX2 could transcriptionally upregulate the expression of CD59, but not the expression of CD46 and CD55, in epithelial CSCs and this mechanism protects cancer cells from complement destruction. After overexpressing SOX2 in MCF-7 and Calu-3 cells a CDC assay was conducted to test the effect of upregulated CD59 by SOX2 functionally; the results demonstrated that SOX2-overexpressing MCF-7 and Calu-3 cells are more resistant than control cells to cetuximab-mediated complement damage [59]. The antitumoral effect of this mAb is abolished by overexpression of CD59 in lung and breast cancer cells, even when this mAb is not used for breast cancer treatment, this mCRP affects its possible repositioning.

Other studies have reported that enrichment of CSCs in the tumor population can confer resistance to therapy, providing worse scenarios for the prognosis of

patients. In tumor cells (CaSki, H1299, HCT116, and HEK293), NANOG increased CD59 expression, contributing to the resistance of tumor cells against complement-dependent cytotoxicity (CDC) [79]. Another example is the role of CD55 in the maintenance of CSCs by regulating self-renewal and cisplatin resistance. CSCs have been implicated in tumor recurrence and treatment resistance, and cisplatin is used for endometrioid tumors and breast cancer. CD55 regulates self-renewal and core pluripotency genes via ROR2/JNK signaling and, in parallel, cisplatin resistance via lymphocyte-specific protein tyrosine kinase (LCK) signaling, which induces the expression of DNA repair genes. Overexpression of CD55 in non-CSCs increased NANOG and SOX2 mRNA levels (core pluripotency genes). It led to significantly higher self-renewal and stem cell frequencies, with lower levels of caspase 3/7 activity upon cisplatin treatment [14].

5.4.2 Signaling pathways

Many studies have established that the transcription factor STAT3 is constitutively activated in various human cancer cells and tumor tissues compared with their normal counterparts. Different signaling pathways involving persistent STAT3 activation have been related to modulating the cancer stem cell phenotype in breast cancer [80, 81]. The role of tumor cell STAT3 signaling in immune evasion has also been described by negatively regulating cellular and innate immune responses [82]. A potential mechanism has been suggested by which oncogenic signaling contributes to tumor cell evasion of antibody-mediated immunity. Buettner et al. demonstrated that activation of STAT3 signaling induces the CD46 promoter and protects human cancer cells from complement-dependent cytotoxicity. Using microarray gene expression profiling, the CD46 gene was identified as a target for activated STAT3 signaling in human breast (MDA-MB-435 s cell line) and prostate cancer cells (DU145 cell line). Moreover, in luciferase reporter assays, CD46 promoter activity was induced by STAT3 activation and blocked by STAT3β, a dominant negative form of STAT3. Finally, inhibition of cell surface expression of CD46 mediated by inhibition of STAT3 signaling sensitized prostate cancer cells to cytotoxicity in an *in vitro* complement lysis assay using rabbit anti-DU145 antiserum, as a source of antibodies, and rabbit complement where cell death was measured by lactate dehydrogenase release [35]. This study shows that STAT3 can contribute to protecting cancer cells from complement system attack, at least through the upregulation of CD46. Still, the regulation of others mCRPs, such as CD55 and CD59, could also be evaluated.

Another mechanism of CDC resistance in breast cancer cells has been described. One study found that Mammalian hepatitis B X-interacting protein (HBXIP), a novel oncoprotein, upregulates mCRPs through ERK1/2/NF-κB signaling to protect breast cancer cells from complement attack [83]. The results showed that HBXIP decreased the sensitivity of MCF-7 cells to CDC; then, CDC susceptibility was rescued when mCRPs were blocked with antibodies against CD46, CD55, and CD59. Furthermore, overexpression of HBXIP was able to upregulate the expression of these mCRPs in levels of promoter activity, mRNA and protein expression in MCF-7 and MDA-MB-231 cells. Finally, the inhibition of ERK1/2 and NF-κB was able to sensitize the MCF-7 cells with HBXIP overexpression to CDC; this was examined by trypan blue absorbance assay after treatment with PD98059 (an inhibitor of MEK) or PDTC (an inhibitor of NF-κB). Thus, the role of HBXIP in regulating mCRPs has been suggested as a complement resistance mechanism in breast cancer cells.

6. Therapeutic strategies and future challenges to regulate mCRP in breast cancer

Here we have exposed that the function of mCRP on complement has been widely explored. But these proteins also can regulate non-complement signaling in promoting cancer proliferation, chemoresistance, and metastasis. We proposed a model of signaling pathways activated by mCRP [12] and recently Bharti et al. also proposed a series of pathways intracellularly by CD55 [43]. Due to their relevance in the potential of anticancer antibodies, they have been proposed and studied mCRP as therapeutic targets through various models and strategies: small interfering RNAs (siRNA) [69, 71, 84, 85], antibodies anti-mCRP [13], and enzyme-peptide [13, 86]. Although there are multiple studies in the preclinical stage and in development, none are exclusive for each mCRP and neither has reached clinical use.

Geis et al. designed siRNAs for post-transcriptional gene knockdown of CD46, CD55, and CD59 aiming to sensitize tumor cells lines (BT474 (breast) and K562 (erythroleukemia) and Du145 (prostate)) to better for tumor cell destruction by complement. Interestingly, the breast carcinoma cells BT474 were predominantly sensitized to CDC upon inhibiting CD46 expression. In contrast, suppression of CD55 and CD59 had no or only a minor effect, suggesting that CD46 is more critical in regulating complement activity [69]. But it is necessary to identify the activity intracellular to all mCRP in this context.

Other authors also used siRNAs anti-mCRP, but the delivery of chemically was stabilized siRNAs using cationic lipoplexes (AtuPLEXes). Their results suggest that siRNA-induced inhibition of mCRP expression enhances complement and macrophage-mediated anti-tumor activity of trastuzumab and pertuzumab on HER2-positive tumor cells [71].

To increase the selectivity of silencing, other authors used siRNAs encapsulated in transferrin-coupled lipoplexes for the specific targeted and delivery to transferrin receptor CD71high expressing BT474 tumor cells. The mCRP knockdown led to a significant increase of CDC in BT474; it was also observed that the downregulation of CD46 and CD55 significantly increased C3 opsonization in these tumor cells [84].

The inhibition of mCRP with siRNA has been used to study the relevance of this molecule in other tumors [87, 88]. In general, silencing can sensitize tumor cells to CDC and ADCC *in vitro*. Although siRNA is an attractive strategy, in vivo data will be needed to validate the therapeutic potential.

Wang et al. explored three different strategies to inhibit the activity of mCRP. One strategy consisted of inhibiting the expression of mCRP using shRNA (short hairpin RNA). Other approach blocked the function of CD55 and CD59 using targeted monoclonal antibodies; the third consisted of treatment with a phosphatidylinositol-specific phospholipase C (PI-PLC), which caused a significant decrease in the surface area of CD55 and CD59. These strategies significantly improved cell lysis of SK-BR-3 and BT474 cells with trastuzumab [13].

The use of monoclonal antibodies anti-mCRP or bispecific antibodies (bsAbs) has also been evaluated in other types of cancer, demonstrating that the efficacy of therapeutic antibodies can be increased by blocking these proteins [89–96].

Ad35K++ that binds with high affinity to CD46 and is one of the most advanced strategies to block CD46 activity. This peptide has been evaluated in lymphoma model and the preclinical studies Ad35K++ have been demonstrated safety and efficacy. Intravenous Ad35K++ injection triggers the shedding of the CD46 extracellular

domain in xenograft mouse tumor models and in macaques. The authors suggest their study is the basis for an investigational new drug application for the use of Ad35K++ in combination with rituximab in the treatment of patients with B-cell malignancies [86, 97]. The first studies with this peptide were evaluated in cancer lines cells (Raji lymphoma cells and BT474-M) using alemtuzumab and trastuzumab, in both case increased CDC [86].

7. Conclusions

The molecular mechanisms of mCRP in the drug resistance of breast cancer cells are still poorly understood. Few investigations have focused in study on the relationship between mCRP and drug resistance. Also, we must learn how mCRP activates intracellular pathways and relate its domains and interactions with other proteins to perform non-complement dependent functions. Therefore, we suggest:

- Future research ought on the role of mCRP in the resistance of breast cancer cells to therapy available, including chemotherapeutic agents and mAb.
- Direct our efforts to understand mCRP signaling in breast cancer and its activity-structure relationship to offer opportunities for regulation or silencing.
- We need rigorous investigation of the pathways activated by mCRP to develop better cancer therapy and improve the efficacy of existing treatments.
- Identify the signaling pathways activated by mCRP. If a previously studied signaling pathway is identified, we could use the drugs developed to regulate the activity of its components. This would lead to new therapeutic applications of the drugs evaluated or to identify the relevance of the signaling pathway activated by mCRP.
- Explorer whether the combined use of tamoxifen and trastuzumab for the treatment of HER2-positive breast cancer enhances the antitumor effects of trastuzumab as suggested the evidence.
- Demonstrate whether therapeutic agents can directly regulate mCRP expression or do so by activating signaling pathways.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Sofia Álvarez-Lorenzo[1,2], Rebeca Elizabeth Montalvo-Castro[1,2], Jeannie Jiménez-López[1,2], María Adriana Medina-Mondragón[1,2] and Nohemí Salinas-Jazmín[1*]

1 Department of Pharmacology, School of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, Mexico

2 School of Chemistry, National Autonomous University of Mexico (UNAM), Mexico City, Mexico

*Address all correspondence to: nohemysj@unam.mx

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