Book Chapter

The Invadopodial Protein CRP2 in Breast Cancer: Molecular Pathology and Therapeutic Perspectives

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Introduction

Cancer is an increasingly challenging global health concern. Out of the most commonly diagnosed cancer types in 2022, breast cancer, lung cancer and colorectal cancer account for 52 % of all

recent diagnoses in women in the United States. Breast cancer accounts for almost the third [1].

Breast cancer is still ahead of all causes of cancer-related deaths in women [2] despite the early diagnostic strategies and the refinement in treatment concepts [3,4]. This goes back to the fact that patients may eventually experience distant recurrence of the disease; a process called Metastasis [5]. Metastasis contributes to 90% of deaths from breast cancer, and no efficient treatment modality exists at the moment that can suppress or even prevent metastasis. As a consequence, a further investigation of the processes by which cancer cells leave the primary site of the tumor and spread to distant organs can likely lead to the development of tailored treatment options as well as the identification of novel prognostic biomarkers [6].

Activating invasion is an early step in the metastatic cascade where primary cancer cells breach the surrounding tissues (epithelial basement membrane and stromal connective tissue) and join the circulatory or the lymphatic system to facilitate their spread and colonization within the body [7,8]. In breast cancer like many other cancer types (melanoma, head & neck and prostate cancers) cancer cells form specialized membrane protrusions known as invadopodia to invade tissue barriers [9]. Invadopodia, first described by Chen in 1989, are rosette-like structures that develop at the basal surface of cancer cells and are active sites for extracellular matrix degradation [10].

Invadopodia, similar to podosomes in normal cells [11], are actin-based protrusions and "hotspots" where proteolytic, cytoskeletal and adhesion proteins converge with key signaling pathways [12]. Structurally, invadopodia are composed of a dense F-actin core surrounded by a protein ring [13]. The F-actin core consists of actin filaments assembled into thick bundles by actin-bundling proteins such as neural Wiskott–Aldrich syndrome protein (N-WASp), actin-related protein 2/3 (Arp2/3) complex, cofilin and cortactin around which a ring of adhesive and scaffolding molecules like integrins, vinculin, and phosphorylated paxillin is formed [13-18]. In addition to that, many signaling molecules synergize to promote invadopodia

formation such as Src family kinases, tyrosine kinase adaptor proteins, phosphatidylinositol, and small GTPases like cdc42 [19]. Invadopodia also constitute a repertoire of secreted and membrane-tethered matrix metalloproteases (MMPs), ADAM family members, the urokinase plasminogen activator receptor (uPAR) and membrane-bound serine proteases [20].

Cysteine and Glycine-Rich Protein 2 (CRP2) is a two-zinc binding LIM domain-containing protein that belongs to the cysteine-rich protein (CRP) family, a family of evolutionarily conserved proteins that mediate protein–protein interactions and are essential for cytoskeletal remodeling, development and transcription control [21]. In metastatic breast cancer, CRP2 is highly expressed, and it localizes along the protrusive actin core of formed invadopodium contributing to the invasiveness of breast cancer cells and to their spread to distant regions within the body [22]. In this review, we will be discussing the role of CRP2 in the human body and in specifically in breast cancer as an invadopodia actin bundling factor in addition to the possible therapeutic possibilities it offers.

CRP2, a Molecular Definition

CRP2 at Gene Level

CSRP2, the gene encoding the LIM domain protein CRP2, belongs to the *CSRP* multigene family. The three genes in this family were independently isolated: *CSRP1* gene was originally identified in human [23], CSRP2 in quail [24], and CSRP3 in rat and chicken [25]. In 1997, Weiskirchen et al. isolated the human CSRP2 (hCSRP2) homolog and determined its entire structural organization; hCSRP2 was mapped to the long arm of chromosome 12 (12q21.1) spanning a total of approximately 22 kb and consisting of six exons of which exons 2–6 defining the coding region of the gene. CSRP2P, a CSRP2-related pseudogene, was also identified and mapped to 3q21.1. hCSRP2 cDNA clone predicted the 193-amino-acid CRP2 protein that shared 96.4% amino acid sequence similarity with its avian homolog [26]. Additionally, CRP2 shares a high sequence identity with CRP1 and CRP3 but has a different spatial and

temporal expression pattern depending on the type of the tissue under consideration [27].

CRP2 at Protein Level

i. CRP2 as a LIM domain containing protein:

CRP2, a cysteine rich protein, belongs to the class of LIM domain proteins that have two tandemly arranged LIM domains, each linked to a short glycine-rich region but lack classical DNA-binding homeodomains [28,29]. A LIM domain (Linl-1, Isl-1 and Mec-3) is 50–60 amino acids in size and has two characteristic zinc finger domains which are separated by two amino acids [30]. Zinc fingers usually function as DNA binding sites, but research has shown that in the case of LIM domains, the zinc fingers function as sites for protein-protein interactions [30].

ii. CRP2 as an actin cytoskeleton binding and bundling protein

CRP2 is found to be present in both the nucleus and the cytoplasm. In the cytoplasm, CRP2 is associated with the actin cytoskeleton where it interacts with F-actin via its N-terminal LIM domain and glycine-rich region facilitating actin polymerization, crosslinking and clustering [31].

Furthermore, numerous actin-binding proteins, such as α-actinin and filamin, regulate the superstructure of F-actin where they form a variety of actin structures including meshworks and networks of thick bundles. Of these actin-binding proteins, CRP2 associates with α-actinin where a study showed that both molecules have close but different interacting positions with F-actin allowing them to act cooperatively to bundle and crosslink actin filaments [31].

iii. CRP2 as a regulator of the cytoarchitecture and migration of vascular smooth muscle cells:

Among the different CRPs, CRP2 is expressed mainly in vascular smooth muscle cells where, in addition to cytoplasmic roles, CRP2 has been reported to form complexes with serum response factor SRF and GATA transcription factors in the nucleus to facilitate vascular smooth muscle cell differentiation [32]. Normally, vascular smooth muscle cells exhibit a quiescent and differentiated phenotype and express proteins involved in the contractile functions such as smooth muscle α-actin, but after an

arterial injury, these cells de-differentiate and downregulate smooth muscle marker genes changing to a proliferative and migratory phenotype to manage the injury [33]. Studies showed that balloon or wire artery injury reduces CRP2 expression, and the lack of CRP2 enhanced vascular smooth muscle cell migration suggesting a critical role for CRP2 in the migration of vascular smooth muscle cells [34,35].

CRP2 and Human Cancers

CRP2 has been shown to play a role in the progression of various types of cancer. In hepatocarcinogenesis, early-stage hepatocellular carcinoma forms small nodules consisting of well-differentiated cancerous tissues. These tissues may occasionally give rise to less differentiated cancerous tissues within the well-differentiated tumor during its progression. Studies show that the upregulation of CRP2 is related to this dedifferentiation of hepatocellular carcinoma [36].

Another study proved that CRP2 is a downstream target of miR-27a which is a microRNA released by gastric cancer cells in exosomes. CRP2 expression is inversely proportional to that of miR-27a in gastric cancer and its downregulation can transform normal fibroblasts into cancer associated fibroblasts which accelerate the progression of the gastric cancer [37].

In colorectal cancer, the overexpression of CRP2 increased the levels of E-cadherin and decreased that of vimentin, β-catenin, and N-cadherin. This means that CRP2 inhibits epithelial-to-mesenchymal transition in these cells which is an important process in cancer invasion and metastasis. Thus, CRP2 has the potential to suppress the invasion and migration of colorectal cancer cells [38].

One study indicates that the knockdown of CRP2 promotes proliferation and cell cycle progression as well as drug resistance in acute myeloid leukemia (AML) cell lines. This knockdown promoted proliferation and cell cycle progression through the regulation of the AKT and CREB pathways. In addition to that, low CRP2 levels were correlated with a relatively higher

cumulative incidence of relapse rate and worse relapse-free survival rate in adults with AML [39].

CRP2 and Breast Cancer

Clinical Relevance of CRP2 in Human Breast Cancer

CRP2's function in crosslinking actin filaments suggests that it contributes to the assembly of the actin-based invadopodia. CRP2 knockdown significantly inhibits invadopodium formation in aggressive breast cancer cells supports that claim [40]. As proof of clinical relevance to human breast cancer, microarray data identified CRP2 in a cluster of 14 upregulated genes characteristic of the highly aggressive basal-like breast carcinoma subtype [41].

Expression and Regulation of CRP2 in Human Breast Cancer Cell Lines

Hypoxia is a common feature of solid tumors and the hypoxic tumor microenvironment is a strong driver of tumor aggressiveness and metastasis, and is highly associated with poor clinical outcomes in various cancers [42]. Hypoxia has recently been reported to promote the formation of the actin-rich membrane protrusions, invadopodia [43]. Furthermore, cis-regulatory elements, termed hypoxia responsive elements (HRE1 and HRE2) where identified two high-confidence HREs in the proximal promoter region of the gene coding for CRP2 [40]. These HREs are binding sites for HIF-1α which is activated by tumor cells in hypoxic conditions [44]. A study showed the effects of hypoxia on the expression of CRP2 in four breast cancer cell lines, including weakly invasive luminal/epithelial-like MCF-7 and T47D (ER+, PR+), and invasive mesenchymal-like MDA-MB-231 and Hs578T (ER−, PR−, HER2−, claudin-low) [40].

i. In normoxic conditions: CRP2 was expressed at significant levels in mesenchymal-like cells whereas it was absent or only weakly expressed in epithelial-like cells. In addition, invasive cells exhibited detectable amounts of HIF-1α under normoxia. This can be attributed to LINK-A37which works

on the stabilization of HIF-1α and activation of HIF-1 signaling in invasive breast cancer cells. This pathway may explain, at least to some extent, the normoxic expression of CSRP2 [40].

ii. In hypoxic conditions: there was a significant up-regulation of CSRP2 in all four cell lines with CRP2 protein levels increased by about ten times in epithelial-like cells and by about five times in mesenchymal-like cells, as compared to the respective normoxic conditions. Additionally, there was a significant and dramatic increase (>12 fold) in HIF-1α occupancy to both HREs compared to normoxic conditions [40]. Figure 1 shows the results from the study where HIF-1α and CRP2 levels were elevated in hypoxic conditions [40].

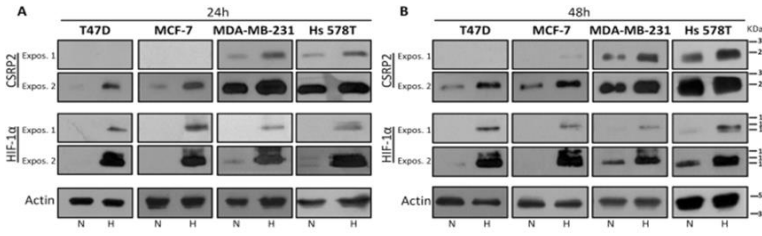

Figure 1: Western blot analysis of CSRP2 and HIF-1α protein levels in the 4 human breast cancer cells (T47D, MCF-7, MDA-MB-231, HS-578T) cultured for 24 h (A) or 48 h (B) in normoxic (N) or hypoxic (H) conditions. Short and long exposures for CSRP2 and HIF-1α blots are shown to better appreciate the differences between the cell lines ("Expos. 1" and "2", respectively) [40].

CRP2 Enhances the Metastatic Phenotype of Breast Cancer Cells by promoting the Biogenesis of Invadopodia

Invadopodia biogenesis largely relies on cytoskeletal rearrangements which are managed by a combination of lamellipodial and filopodial actin machineries [45]. The assembly of an actin core by the ARP2/3 complex and its associated regulators, such as N-WASP and cortactin, is a crucial step of invadopodium initiation. Invadopodium elongation is then promoted by the expansion of the actin core in both branched networks and unbranched bundles [16]. In the core,

actin filaments are cross-linked in thick bundles, which presumably focus actin polymerization-promoted force for protrusive activity, and stabilize invadopodia over long periods to optimize extracellular matrix degradation by metalloproteinases (MMPs) [22].

CRP2, being an actin -binding protein, plays a key role in invadopodia biogenesis where it can change actin polymerization from a randomly organized meshwork of fine actin filaments into reticulated network of thick and long actin bundles [22]. CRP2 has also been proven to localize with the actin fibers in the invadopodia of breast cancer [22].

In invasive breast cancer, hypoxia promoted invadopodia-mediated extracellular matrix degradation where studies showed that the percentage of active cells associated with local ECM degradation increased from about 50% in normoxia to about 70% in hypoxia. Additionally, hypoxia induced a 5-fold increase in the average surface of matrix degradation [40]. The stimulatory effects of hypoxia were inhibited by CSRP2 knockdown which decreased the percentage of active cells, the degradation index and the number of invadopodia per cell to values similar to those obtained for control cells in normoxia [40]. In support of this, the invasiveness of HIF-1α depleted invasive breast cancer cells was increased by CSRP2 forced expression under hypoxia [40].

Furthermore, even though weakly invasive breast cancer cells fail to promote extracellular matrix degradation, they were able to give rise to invadopodia precursors through CRP2 in hypoxic conditions [40].

CRP2 Transcriptionally Activates Pro-Metastatic Matrix Metalloproteinases (MMPs)

Extracellular matrix degradation is primarily mediated by metalloproteinases (MMPs) that are secreted at sites of invadopodia. And among secreted MMPs, the gelatinases MMP-2 and MMP-9 are repeatedly associated with breast cancer progression [22]. Invasive breast cancer cells, which secrete low

basal levels of MMP-2 and MMP-9, have been shown to respond to phorbol 12-myristate 13-acetate (PMA) by secreting MMP-9 [46]. Membrane type-1 MMP (MT1-MMP) is a membrane-tethered MMP that catalyzes MMP-2 activation by cleavage of its pro-domain34, and that MMP-2 contributes to MMP-9 activation [47]. In weakly invasive breast cancer which lacks MT1-MMP and can't perform extracellular matrix degradation, CRP2 gave rise to invadopodium precursors in hypoxic conditions with increased secretion of mostly inactive, high-molecular weight, forms of MMP-2 and MMP-9 suggesting that, although proteolytically inactive, these invadopodium precursors are mature enough for MMP secretion [40].

CRP2 as a Therapeutic Target in Breast Cancer

Use of MMPs Inhibitors: Failed in Clinical Trials

The extracellular matrix-degrading activities of MMPs in metastatic disease, especially in highly aggressive late-stage tumors with poor clinical outcome, made them an attractive cancer treatment target [48]. Preclinical studies testing the efficacy of MMP suppression in tumor models were so compelling that synthetic metalloproteinase inhibitors (MPIs) were rapidly developed and routed into human clinical trials [49].

Early phase I clinical trials revealed that prolonged treatment with MPIs caused musculoskeletal pain and inflammation, complications not seen in preclinical models. These side effects were reversible after taking breaks from the medication but they limited MPI dosages administered in subsequent trials [49].

Phase II trials which are designed to examine efficacy were problematic as well, since MPIs are not cytotoxic (cells are killed) but rather cytostatic (cells are growth-arrested but viable). This meant that conventional measures of efficacy such as reduction in tumor size could not be used to monitor drug activity [49]. As a consequence of these and other issues, phase I trials were followed immediately by phase II/III combination trials without the benefit of efficacy information from smaller studies [49].

Phase III trials are large-scale studies that evaluate efficacy in comparison to standard treatments. These trials examined the efficacy of the MPI alone versus that of cytotoxic drugs and the effect of an MPI, either in combination with or after treatment with cytotoxic drugs, compared with the effect of the cytotoxic drugs alone. The results of these trials have been disappointing and many investigators concluded that MPIs have no therapeutic benefit in human cancer [49].

Targeting Invadopodia as an Alternative for Blocking Breast Cancer Metastasis

The failure of MMP inhibitor-based strategies makes targeting invadopodia an attractive alternative [22]. In this context, CRP2 emerges as a new potential therapeutic target to treat metastatic breast cancers [22]. As mentioned before, studies have shown that CRP2 knockdown inhibits extracellular matrix degradation and MMP-9 expression and inhibits metastatic colonization [22]. In addition, one study found that mice lacking CRP2 are viable, fertile and only exhibit subtle alteration of cardiac ultrastructure [34]. This means that it is possible that targeting CRP2 in patients would only cause minor side effects [22].

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