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CD44 as a Breast Cancer Stem Cell Marker

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Abstract: Breast cancer in women is a major public health problem throughout the world. It is the most common cancer among women both in developed and developing countries. Breast Cancer is ranked as the first malignancy affecting females. The cancer stem cell (CSC) model proposes that tumors are organized in a cellular hierarchy, in which CSC are the only cells with unlimited proliferation and tumorigenic potential; therefore, being capable of driving tumor growth, progression and metastasis due to their stem cell-like characteristics: self-renewal and differentiation**.** CSC is resistant to various forms of therapies, including radio and chemotherapy. Based on these observations, the CSC model became the foundation for new preventive and therapeutic strategies in cancer. In breast cancer, CD44 is positively associated with stem cell-like characteristics**.** Interestingly, CD44+ cells are more frequently found in basal-like than in luminal tumors. Based on this knowledge, there is evidence to support the idea that the use of CD44 cell surface marker is an accurate method to identify and isolate CSC-like cells within breast cancer populations.

Key words: Breast cancer · Cancer stem cell · CD44

INTRODUCTION

Breast cancer in women is a major public health problem throughout the world. It is the most common cancer among women both in developed and developing countries. Breast Cancer is ranked as the first malignancy affecting females [1], there were 1.8 million new cases and 464,000 deaths in 2013. Breast cancer caused 13.1 million disability-adjusted life-years (DALYs) in 2013, with 63 percent occurring in developing countries and 37 percent in developed countries. One in 18 women developed breast cancer between birth and age 79 [2].

CD44, especially CD44v isoforms, have been identified as CSC markers and critical players in regulating the properties of CSCs in many types of tumors. With better understanding of the fundamental basis of how CD44 and CD44v expressions are regulated and identification of novel and unique CD44v isoforms in CSCs, effective therapeutic strategies that aim to eliminate CSCs by targeting CSC-specific CD44 isoform(s) may be developed that will bring new hope to patients with life-threatening cancer. [3]

Currently, this review was focused on characterizing a specific marker that can identify high-risk patients and have the potential to be developed as a targeting strategy for future therapies.

Cancer Stem Cells: Tumors are proposed to be organized in a cellular hierarchy, in which CSCs are the only cells with unlimited proliferation and tumorigenic potential; therefore, being capable of driving tumor growth, progression and metastasis due to their stem cell-like characteristics: self-renewal and differentiation [4].

According to the cancer stem cell hypothesis, both hereditary and sporadic cancers can arise from deregulation of these cancer stem cells (CSCs), triggered by genetic and environmental factors [5].

Understanding the bio-mechanism of stem cell metabolism and its regulation by signaling molecules and extracellular micro-environment is an important step toward a successful prevention and treatment of cancer [5].

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Stem cells are localized in a niche which is a local tissue microenvironment. The niche is formed by a group of cells (fibroblasts of the stroma) which have a support function for stem cells and physical adhesion molecules that mediate interactions between the stem cells, the support cells and the extracellular matrix. When there is a change in the niche and growth and proliferation signals prevail, the stem cell population is exposed to an uncontrolled expansion which can lead to spread of CSCs [6].

Cancer stem cells are slowly proliferating cells, quiescent in G0 phase for long periods of time; hence they may escape conventional treatments that mainly target actively proliferating cells. CSCs have similar self-renewal properties to normal stem cells; however the balance between the signaling pathways is altered towards tumor formation [4]. CSCs are not necessarily transformed normal stem cells; they may arise from restricted progenitors or differentiated cells by acquiring stem cell-like properties and further undergo clonal selection to generate different subtypes of breast cancers (Triple negative, Her2-gene amplified, luminal) [7].

CSCs have been detected in primary and metastatic tumors, where they sustain the tumor mass, preserve its heterogeneity and drive metastasis [8, 9].

Breast Cancer Stem Cells: Human breast cancer stem cells (BCSCs) can be generally divided into two subcategories, one expressing the $ALDH1^+$ and the other displaying the $CD44^+/CD24^-$ phenotype. These have been shown to interconvert from one type to another, presumably depending on the tumor phase and requirements [10]. BCSCs have also been found in the peripheral blood of breast cancer patients [11,12] where they promote systemic dissemination in the bone marrow of early stage breast cancer patients [13].

CD44 as a Cancer Stem Cell Marker: CD44 is highly expressed in many diseases. It has been found in cancerous, inflammatory and auto-immunological diseases. It has been identified as a typical CSC surface marker, individually or in combination with other marker(s) [14].

It was found that the CD44 levels were higher in many tumor malignancies, it promotes homing of CSCs in many types of solid tumors and high CD44 expression is closely correlated with enhanced spheroid colony formation in many cancers including breast cancer [15-17].

CD44 is a transmembrane glycoprotein that participates in many cellular processes including regulation of cell division, survival, migration and adhesion [18], through the binding of its major ligand, hyaluronic acid (HA) and by acting as a cellular platform for growth factors and heparan sulphate proteoglycans. It can also act as a co-receptor to mediate signaling of the HER-2 family and MET receptor tyrosine kinases, possibly by organizing the assembly of functional complexes. CD44 also provides a link between the plasma membrane and the actin cytoskeleton, modulating cellular shape and motility [19]. The human CD44 gene is located on chromosome 11p13 and consists of 19 coding exons of which 9, residing between constitutive exons 5 and 6, can be alternatively spliced into many different isoforms with tissue and differentiation specific expression. The standard isoform of CD44 (CD44S) contains none of the 9 variable exons, whereas the CD44v2-v10 isoform includes them all (exon v1 is not expressed in humans). The CD44v3-v10 isoform has one less exon and the CD44v8-v10 isoform includes only the last three of the variable exons [20].

The CD44 molecule consists of an amino-terminal extracellular and ligand-binding domain, a membrane proximal stem loop including the variable region, a transmembrane region and a cytoplasmic tail that attaches to actin and ankyrin in the cytoskeleton [20]. The epitope recognized by the CD44 antibodies commonly used for isolation of cancer stem cells (CSCs) is situated in the amino-terminal region of CD44 consisting of the non-variable exons 1 to 5, indicating that all CD44 isoforms should be detected by this antibody [19].

Pathological role of CD44: CD44 expression is regulated by many extracellular or intracellular factors during tumor development. Activation of STAT3 (Signal transducer and activator of transcription 3) signaling promotes stem cell-like traits by upregulating CD44 and a feedback regulation between STAT3 and CD44 is observed. Interleukin-6 exposure activates interleukin-6/STAT3 signaling in CD44 (-) tumor cells and induces upregulation of CD44 protein expression, resulting in the enrichment of CD44 (+) cell population [21].

In breast cancer, CD44 promotes the phosphorylation of STAT3 by interacting with STAT3 and then the pSTAT3 moved to nucleus and combined with NF-êB to activate hTERT (human telomerase reverse trascriptase), which in turn increases CD44 expression [22]. Transforming growth factor â1 (TGF-â1) up regulates CD44. In agreement with these findings, blocking TGF-â1 signaling in these cells decreases CD44 expression [23,24]. On the contrary, CD44 knockdown resulted in much reduced spheroid colony formation and smaller tumor production and the $CD44^-$ populations had significantly reduced tumorigenic ability in vitro and in vivo [25].

Given that many CD44v isoforms are preferentially expressed on cancer cells and required for tumorigenesis and progression, CD44v isoforms presumably might be better CSC markers than the CD44s isoform [26-28].

CD44 plays pivotal roles in promoting tumor invasion and metastasis by contributing to adhesion of tumors cells to endothelium and fibronectin-enriched matrices [29]. CD44v possesses E-selectin ligand activity; expression of CD44 in breast cancer cell enhances adhesion to endothelial cell and correlates with metastasis potential [30]. CD44 potentiated the adhesion of basal-like breast cancer cell to endothelium and fibronectin in an alpha5B1-integrin-dependent manner [29].

Metastatic breast tumors expressing the hyaluronan receptor CD44 are associated with high grade and are correlated with increased distant recurrence and reduced disease-free survival in patients with lymph-node positive or large tumors. To determine its functional role in distant metastasis, CD44 knock down attenuated tumor cell adhesion to endothelial cells and reduced cell invasion but did not affect proliferation in vitro. CD44 knock-down increased survival and decreased overall tumor burden at multiple sites, including the skeleton in vivo. Elevated CD44 expression on tumor cells within the systemic circulation increases the efficiency of post-intravasation events and distant metastasis in vivo, consistent with its association with increased distant recurrence and reduced disease-free survival in patients [29].

CD44 plays an important role in different subtypes of breast cancer. It might acquire an oncogenic signaling pathway since it has been found interfering with the expression of well-known oncogenic markers such HER2 (human epidermal growth factor receptor 2), ER (estrogen receptor) and PR (progesterone receptor) [31]. Further, a study demonstrated that CD44 variants were heterogeneously expressed in breast cancer and correlated with tumor subtypes and cancer stem cell markers. They showed that a high expression of CD44v2–10 isoform, which retain all variant exons, was correlated to positive steroid receptor status, low proliferation and luminal A subtype. The CD44v3–10 showed similar correlation, while high expression of CD44v8–10 was correlated to positive EGFR, negative/low HER2 status and basal-like subtype. Further, the CD44 variants were associated with all tumors that were characterized as positive for $CD44^{+}/CD24^{-}$ phenotype by immunohistochemistry. These findings suggested the involvement of CD44 variants in specific oncogenic signaling pathways [31].

Relation between epithelial mesenchymal transition (EMT) and CD44: Mesenchymal stem/stromal cells (MSC) are a population of multipotent stem cells that exist in most tissues of the body. Under circumstances of growth or repair, these resident progenitor cells repopulate the tissue and provide structural support and paracrine stimulation. They are known to migrate toward inflamed/injured environments, including tumors [32] and can be induced to express markers associated with tumor/cancer-associated fibroblasts (TAF/CAF) and myofibroblasts [21]. Stromal cells of mesenchymal phenotype fill the interstitial space of tumors and provide growth factors, matrix remodeling factors and other tumorigenic supportive factors [33]. They are often referred to as activated (myo) fibroblasts or tumor associated fibroblasts (TAF) and has been shown to originate from multiple sources including local tissue–derived fibroblasts, local tissue–derived MSC, bone marrow–derived MSC and cancer cells through the epithelial-to-mesenchymal transition (EMT) [34].

EMT, a tightly regulated and highly conserved cellular process for a cell type changing from an epithelial phenotype to a mesenchymal phenotype, results in the cell acquiring fibroblast-like properties. It plays a crucial role especially, in tumor proliferation, invasion, metastasis, recurrence and drug resistance. [35, 36].

EMT promotes CD44 expression. Mesenchymal genes, such as TWIST1 (Twist Family BHLH transcription factor 1) [37], SNAI1 (Snail Family Zinc Finger1) [38], ZEB1 (Zinc Finger E-box Binding Homeobox 1) [39] and SLUG (Slug Homolog Zing Finger protein) are positively correlated with CD44 expression [40].

Expression of CD44 in mesenchymal cells was increased in advanced breast tumors, showed that the expression of CD44 in mesenchymal cells promoted EMT by activating PI3K/Akt signaling pathways [41]. The CD44- ligand HA binding regulates stem cells homing. Platelet derived growth factor stimulates MSCs to produce more CD44 that facilitates cell traveling through extracellular HA binding. This process is suggested to help in recruitment of MSCs during tissue development and healing. CD44/HA binding seemed to interact with different intrinsic niche factors. Growth factors mediate CD44 to link to TRK to work as cellular signal transducers. [42].

Assessment of CD44 Immunostaining

CD44 Immunostaining in Tumor Cells: Membranous / cytoplasmic reaction is considered positive and the proportions of positive cells are detected as follows:

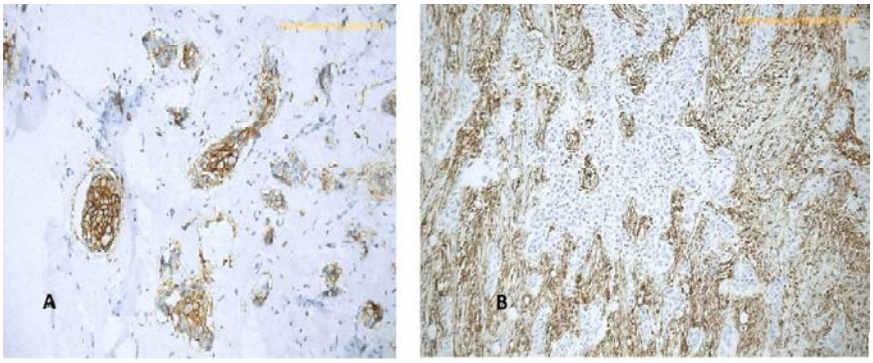

Fig. 1: (A) CD44 membranous and cytoplasmic staining (3+) in the invasive tumor cells as well as tumor emboli (original magnification X200). (B) CD44 stromal immunostaining (original magnification X100). [44].

0 = 0% positive tumor cells, 1 = 1-10% positive tumor cells, 2 = 11-50% positive tumor cells, 3 = 51-75% positive tumor cells, 4 = 76-100% positive tumor cells [31].

CD44 Immunostaining in the Stromal Cells: Expression of CD44 in the stromal cells (i.e., fibroblasts, myofibroblasts and endothelial cells) was graded according to the percentage of positive cells as follows: negative (0–5%); weak (6–25%); moderate (26–50%); strong (51–75%) or very strong (76–100%) [43]. (Fig. 1).

Therapy: CD44 has a role in chemotherapy resistance and relapsing of breast cancer. This indicates an intrinsic chemical resistance of cells has a high expression of CD44 [45]. Many reports support that functional inhibition of CD44 at gene or protein level reverses some malignant behaviors and sensitizes to therapy [46, 47].

Since the discovery that HA receptor, CD44 is a stem cell marker, targeting of CD44 for anti-cancer therapy has been attempted using DNA vaccines, nanoparticle-mediated delivery of CD44siRNAs and anti-CD44 monoclonal antibodies. In addition, HA-coated nanoparticles or anti-CD44 conjugates have been used to target $CD44^{+}$ cells for therapy [48].

CD44 Vaccines: CD44 cDNA or targeting of CD44-expressing cells has been used to generate tumor immunity in experimental models. A recent study has examined the concept of "foreignizing" tumor cells by specifically delivering foreign antigens to target $CD44^{hi}$ tumor cells using a polymeric ovalbumin (foreign antigen) and HA delivery system [49].

Vaccination was achieved by injection of virtual lymph nodes loaded with human CD44 variant (v3–10) or CD44-standard cDNA. Immunized animals expressed antibodies against human CD44 variant and CD44-standard forms. The vaccination against CD44 variant (v3–10) was more effective than vaccination with the CD44-standard isoform in eliminating tumor growth in 75% of the vaccinated mice and slowed tumor growth in the remaining animals. Furthermore, metastasis was eliminated in all animals. Since CD44-standard form did not generate the same immunological response against tumors, it suggested that CD44 variant (v3–10) and not CD44s was functional in promoting tumor growth and metastasis in DA3 cells [50].

CD44 siRNA Delivery: CD44 has been targeted for therapy using specific siRNAs. A challenge with this approach is the alternatively spliced isoforms of CD44. Based on the sequence, the siRNAs may downregulate only certain CD44 variants. The most common isoforms targeted by siRNAs are CD44-standard and CD44v6. Most commonly these CD44 siRNAs have been delivered to tumor cells using nanoparticles [51].

Targeting CD44 for Delivering Antitumor Therapies: Since CD44 is overexpressed in a variety of tumor cells, HA-coated self-assembling nanoparticles or liposomes have been tested for the delivery of siRNAs and/or chemotherapy drugs in pre-clinical xenograft models. The siRNAs reported so far include those specific for the multidrug resistance (MDR) protein or proteins in the apoptosis pathway (e.g., bcl-2, survivin). The advantage of CD44-targeting HA-coated nanoparticles for siRNA delivery is that these nanoparticles are biodegradable and reasonably specific to tumors [52,53]. For the delivery of anti-cancer drugs such as doxorubicin, a photochemically triggered cytosolic drug delivery system based on combining pH-responsive HA nanoparticles containing doxorubicin has been developed [49].

Targeting of CD44 Protein: Anti-CD44 antibodies have also been evaluated as an anti-cancer therapy [54, 55]. In addition to the therapeutic uses of various anti-CD44 antibodies, a study has used an anti-CD44 antibody for tumor imaging. For example, a chimeric monoclonal antibody U36 and its F(ab')2 (Fragment Antigen Binding) and Fab' fragments that recognize the CD44v6 isoform have shown potential for radioimmuno-therapy and radioimmuno-targeting of experimental tumors [56].

As in the case of HA-coated nanoparticles or liposomes, anti-CD44 antibody–drug conjugates have also been used either for imaging of tumors or for delivering chemotherapeutic agents to experimental tumor models. For example, anti-CD44 antibody conjugates have been used to deliver radioisotopes or mertansine for the treatment of CD44-expressing tumors. Disease stabilization was observed in breast or head and neck tumor patients; however, dose-limiting toxicity was observed along with the distribution of the antibody in the skin, where high levels of CD44 are expressed [57]. In addition, the HA-coated nanoparticles may also deliver the cargo to tissues, which express other HA receptors. Therefore, treatment and imaging strategies that target CD44 will have to be carefully evaluated for their effects on normal cells and the immune system. Furthermore, the risk versus benefit must be carefully evaluated before CD44-targeting strategies are translated to the clinic [48].

CONCLUSIONS

CD44 has been identified as a CSC marker and a critical player in regulating the properties of CSCs in many types of tumors, which are the only cells with unlimited proliferation and tumorigenic potential; therefore, being capable of driving tumor growth, progression and metastasis due to their stem cell-like characteristics: self-renewal and differentiation. CD44 has a role in chemotherapy resistance and relapsing of breast cancer. Targeting of CD44 for anti-cancer therapy has been attempted using DNA vaccines, nanoparticle-mediated delivery of CD44siRNAs and anti-CD44 monoclonal antibodies. In addition, HA-coated nanoparticles or anti-CD44 conjugates have been used to target $CD44^+$ cells for therapy. We believe CD44 requires further investigation for use as a successful marker in the early detection of multiple cancer cell lines.

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