Review Article

The Significance of the Variable Region of the CD44 Antigen in the Diagnosis and Treatment of Breast Cancer

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ABSTRACT

In the field of cancer biology, elucidating the factors that promote cancer cell invasion and progression is crucial for the identification of novel therapeutic targets. CD44 has emerged as a vital biomarker with significant implications for innovative treatment modalities, particularly in immunotherapy. This receptor is predominantly expressed on cancer stem cells, which play a pivotal role in tumorigenesis and contribute to therapeutic resistance. Elevated levels of CD44 are associated with aggressive cancer phenotypes, including enhanced metastatic potential and increased resistance to chemotherapy. Therefore, targeting CD44 may enhance the efficacy of cancer treatments. Notably, CD44 exists in multiple isoforms, each exhibiting unique expression patterns across different tissues and types of cancer, highlighting the need for a refined approach to clinical targeting. The variations among these isoforms can influence tumor behavior and treatment responses, underscoring the importance of understanding these distinctions for the optimization of therapeutic strategies. This review aims to delineate key aspects of breast cancer, emphasize the significance of the CD44 biomarker, and explore the implications of its isoforms in diagnosis and treatment. By scrutinizing these elements, we seek to clarify the role of CD44 in cancer biology and its potential for enhancing treatment approaches in breast cancer and other malignancies.

Keywords: Breast Cancer, Cancer Stem Cells, CD44 Variable Region, Diagnosis, Treatment.

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Introduction

One of the most prevalent cancers in women globally and a major contributor to cancer-related mortality is breast cancer (1). It develops in the breast tissue and may present with symptoms such as a breast lump, changes in breast appearance or skin texture, nipple discharge or inversion, or redness and dryness of the breast skin. In advanced metastatic breast cancer, patients may experience bone pain, enlarged lymph nodes, shortness of breath, or jaundice as the cancer spreads to distant organs. The main risk factors for breast cancer include female gender, lack of physical activity, obesity, alcohol consumption, menopause, ionizing radiation, early menstruation, late or no pregnancy, previous breast cancer, and hormone replacement therapy (2). Although inherited mutations in high-risk genes only make up a small portion of cases, environmental factors play a key role in numerous adult cancers, potentially interacting with multiple genetic factors (3). Cancers that originate in the milk ducts are known as ductal carcinomas, while those that originate in the small milk-producing sacs are referred to as lobular carcinomas. In addition, there are around 18 subtypes of breast cancer, including "ductal carcinoma in situ," which is due to the growth of precancerous lesions (4). There are various stages of breast cancer ranging from 0 to 4. These stages are determined by the extent of cancer cell growth and invasion. Stage 0 indicates no metastasis, while the fifth stage represents the most advanced and severe growth of cancer (5). Breast cancer is diagnosed through a biopsy of suspicious tissue. Once the diagnosis is confirmed, additional tests may be conducted to determine if the cancer cells have metastasized beyond the breast, aiding in the selection of the most effective treatment (6). For this purpose, immunotherapy is one of the newer and more effective treatment techniques that aim to enhance the specificity and efficacy of treatment. In immunotherapy, crucial markers are identified, such as CD44, which exhibits elevated expression levels in breast cancer stem cells (BCSC) (7). CD44 serves various functions in physiological processes such as hematopoiesis, the immune system, and organogenesis, as well as in pathological circumstances like cancer and metastasis (8). The CD44 marker is a widespread cell surface receptor and cell adhesion molecule. Alternative splicing of variable exons occurs in CD44 mRNA, leading to the generation of various CD44 isoforms, each with distinct biological functions (9, 10).

For the first time, Suzuki and colleagues demonstrated varied CD44 expression across meningioma subtypes. Multiple studies also indicate that CD44 overexpression is linked to heightened migration and anaplasia in these cells (11, 12). One reason for these capabilities is CD44's interaction with the cytoskeleton via the proteins Ezrin, Radixin, and Moesin, which are structurally similar to the Merlin protein. Morrison et al. also provided further evidence for Merlin's role in the inhibition of cell growth through CD44 interaction in schwannoma cell lines (13, 14). Research indicates that in cases of breast cancer, this biomarker experiences a significant increase within stem cells. The extent of this increase differs based on the particular CD44 isoform. Broadly, the CD44 marker consists of three general parts: an intramembrane part, a cytoplasmic part, and an extracellular part. Within the extracellular domain, different sequences appear at various locations, causing the formation of distinct isoforms (15). New and personalized therapies attempt to exploit these differences, such as through the production of monoclonal antibodies (16). This review study investigates the impact of the CD44 biomarker on breast cancer, specifically focusing on the variable region of the extracellular domain. Enhancing our comprehension of this domain could improve the efficacy of breast cancer treatment, offering valuable insights for future researchers in this area.

Method

Method For this review, a comprehensive literature search was conducted using the scientific databases Scopus, PubMed, and Google Scholar. Relevant articles published up until December 2024 were identified and carefully analyzed based on specific keywords related to CD44 and breast cancer. The selection process aimed to capture the most significant research findings, ensuring a well-rounded discussion of CD44's role in breast cancer progression, diagnosis, and potential therapeutic targets.

CD44 Structure and Function

CD44 is a non-kinase transmembrane glycoprotein weighing 90–220 kilodaltons. It is encoded by a gene on the short arm of chromosome 11 in humans and chromosome 2 in mice, and is also referred to as P-glycoprotein 1 (Pg1). CD44 is an acidic molecule, strongly charged by sialic acid (isoelectric point = 5.4 to 8.5), with a half-life estimated at around 8 hours (17). The CD44 gene consists of 19 exons in humans and 20 exons in mice. The initial and final five exons remain consistent across both species, while exon six lacks similarity in humans (18). Standard CD44 (CD44s) is formed from exons 1–5, 16–18, and 20, with exon 19 typically excluded due to alternative splicing, leading to a truncated tail in most CD44 isoforms. Variable CD44 isoforms (CD44v) arise from the alternatively spliced exons 6–15 (v1–v10), though exon v1 is absent in humans due to an in-frame stop codon. The first variable exon in human CD44 is designated as v2. CD44 variant isoforms may include one or more of these variant exons, such as CD44v6 or CD44v3–10 (19). As shown in Figure 1, CD44 features four distinct domains: the N-terminal HA-binding module, the stem region, the transmembrane domain, and a

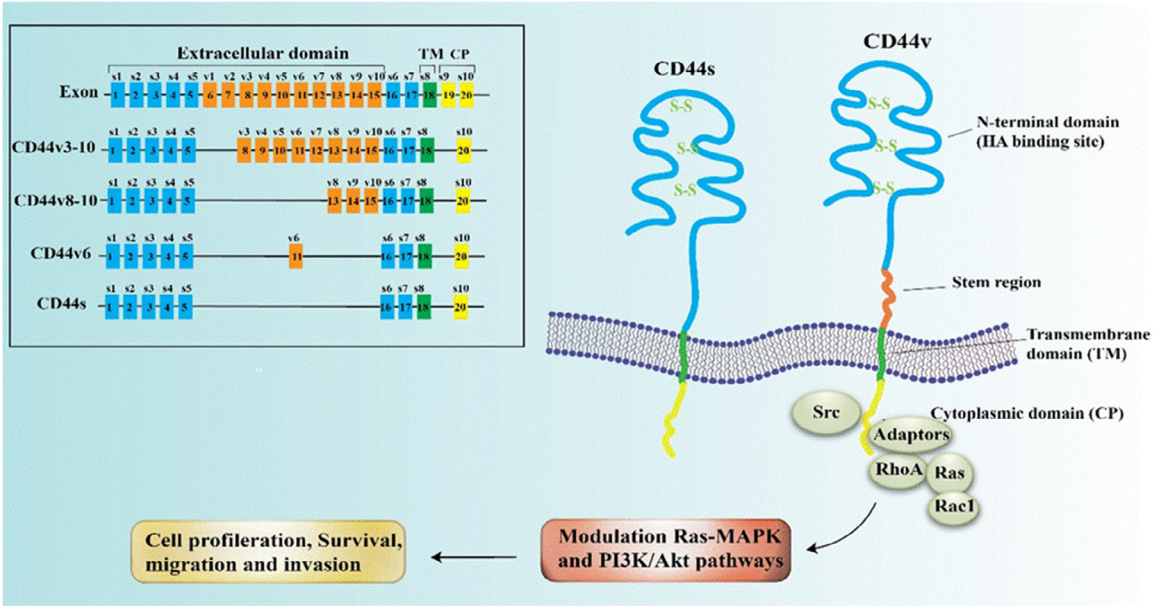

Figure 1. Schematic Representation of CD44 Structure and Function. This diagram illustrates the structural features of the CD44 gene and protein (20).

short C-terminal cytoplasmic domain. The interaction between CD44 and hyaluronic acid (HA) plays a pivotal role in tumor progression. The cytoplasmic tail of CD44 associates with various cytoskeletal proteins, including ERM proteins and ankyrin, facilitating cytoskeletal rearrangement and HA-mediated tumor cell functions. CD44–HA binding also triggers signaling pathways, including activation of Src family non-receptor kinases and various GTPases (e.g., RhoA, Ras, and Rac1). This interaction is crucial for initiating downstream signaling cascades, notably the Ras–mitogen-activated protein and phosphoinositide 3-kinase/Akt pathways, which promote key tumor cell behaviors such as proliferation, survival, migration, invasiveness, and resistance to chemotherapy (20).

Functioning as a receptor for various extracellular components, CD44 binds not only to hyaluronic acid (HA) but also to osteopontin (OPN), matrix collagen, and metalloproteinases (MMPs). It plays a role in attachment, cell migration, invasion, and metastasis (21). It is a marker for cancer stem cell (CSC) detection in numerous tumors, such as breast tumors. Moreover, the role of CD44 in maintaining the function and foundation of CSCs and recurrence has been confirmed, indicating this antigen's significance as a prognostic marker. Studies also show that the expression of CD44 is positively related to the mesenchymal phenotype and metastasis in various cancer types. Furthermore, CD44 is crucial in activating cancer cell survival pathways and evading apoptosis (15, 22). As a result, the CD44 molecule serves not only as a marker for cancer stem cells but also plays a direct role in carcinogenesis and tumor progression. This is evident in mice, where disrupting the CD44 marker gene can lead to a reduction in diverse biological functions to just two molecular action mechanisms (15, 17). First, the CD44 marker can be activated or regulated by ligand binding. This interaction involves adhesion to hyaluronan and other elements of the extracellular matrix, as well as inhibiting the action of cells like axons, serving as a scaffold for enzymes and growth factors (23). Second, the CD44 marker may have co-receptor functions that facilitate receptor tyrosine kinase signaling. This role is significant for a subset of receptor tyrosine kinases, particularly those in the ERBB family (24). These two molecular activities help us understand how CD44 signaling proteins act in a switch between promoting and arresting cell growth and why they induce metastasis in some tumors but not in others. Thus, CD44, a prevalent glycoprotein on the cell membrane, significantly influences various cell actions such as adhesion, migration, invasion, and survival (25).

Specifications and use of CD44

Role of CD44v in Cancer Progression and Tumorigenicity

The CD44v antigen, generated through alternative splicing of the CD44 gene, results in the incorporation of variable exons. These modifications in the extracellular domain lead to the formation of diverse ligand-binding sites and distinct functional traits. Typically found on the surface of several solid tumors such as breast, colon, pancreatic, and ovarian cancer, CD44v expression is linked to poor prognosis and resistance to chemotherapy and radiation. Furthermore, CD44v-positive CSCs exhibit greater tumorigenicity and aggressiveness compared to CD44v-negative CSCs. While CD44v

antigen expression is tightly controlled during embryonic growth, its dysregulation in cancer contributes to the development of CSC characteristics (17, 26). CD44v interacts with different components of the extracellular matrix, like hyaluronan, osteopontin, and collagen, and with intracellular signaling molecules such as Src, PI3K, Rho GTPases, and Wnt/β-catenin. Moreover, epigenetic modifications, microRNAs, and transcription factors also have a significant role in modulating its expression (27). Understanding these influencing mechanisms can provide potential targets for therapeutic interventions. Considering the role that CD44v has in CSCs, it can emerge as a possible therapeutic target for the treatment of cancer. Several approaches have been developed to target CD44v, including monoclonal antibodies (28). Preclinical studies indicate that directing focus toward CD44v has the potential to restrict CSC characteristics and enhance tumor sensitivity to chemotherapy and radiotherapy. Importantly, CD44 isoforms differ depending on variable exons, so treatment and targeting are disease-specific. Furthermore, these isoforms serve as valuable diagnostic indicators (22, 29).

CD44 Isoforms and Their Functional Significance

The CD44 receptor is an approximately 85–90 kDa cell surface glycoprotein that exists in both standard and alternatively spliced isoforms. CD44s lack variant exons and represent the basic CD44 structure generated without splicing alterations (30).

CD44 expression and glycosylation exhibit heterogeneity depending on cell type. It contains seven Asn-linked glycosylation sites in the extracellular region and four Ser-Gly motifs in the membrane-proximal domain that facilitate the binding of glycosaminoglycans like chondroitin sulfate. Additional Ser/Thr residues may also undergo O-glycosylation. CD44v, containing inserted exons, typically displays elevated glycosylation levels through extra Asn- and O-linked glycosylation sites contributed by the variant exon products. Thus, alternative splicing and subsequent post-translational modifications regulate CD44 structural and functional diversity in a cell context-dependent manner. Furthermore, isoforms with v3 and v10 exons offer more binding sites on the GAG side (30). CD44v isoforms, by inserting a variable exon or fusing with other variable exons, can encode peptides near the membrane in the extracellular domain region. Overexpression of certain CD44 isoforms can markedly enhance aggressiveness. Tumor metastasis and cancer cells are strongly correlated (17). Numerous studies indicate that the interaction between hyaluronate and CD44v is tightly controlled. Various mechanisms, such as alternative splicing of exons and post-transcriptional modifications like glycosylation alterations, play a role. CD44v can accommodate up to 13 peptide units in its extracellular domain, each being genetically encoded. This characteristic highlights how variations in glycosylation levels within the genomic sequence contribute to CD44's status as one of the most diverse membrane surface molecules (31). On the other hand, this structural diversity forms the molecular basis that can account for the variety of cellular functions associated with CD44 molecular variants. The functional significance of CD44 isoforms in breast cancer is summarized in Table 1.

Diagnostic and Therapeutic Potential of CD44 Isoforms

There is mounting evidence indicating the significant involvement of CD44 and its splice variants in the growth and progression of cancerous tumors. For instance, v6 has the potential to trigger metastasis in mouse mammary glands. Altered CD44 gene activity in cancer cells results in quantitative and qualitative changes in CD44v isoform expression compared to normal cells. Several studies have reported detectable levels of soluble CD44v molecules in the serum of patients with different types of cancer. This suggests the potential utility of CD44 and its isoforms as non-invasive biomarkers for cancer diagnosis and prognosis. Measuring CD44v levels in patient serum samples and tumor tissues could provide a reliable indicator of malignancy and help predict metastatic potential. If validated in large clinical cohorts, CD44-based liquid biopsy assays may aid early cancer detection, as well as monitoring of disease progression or treatment response over time. This could improve clinical management by facilitating timely diagnosis and interventions before tumors become advanced. Despite limited knowledge regarding the expression and functions of distinct isoforms, it is suggested that specific isoforms may contribute to cancer metastasis (17, 32). A comprehensive analysis of CD44 isoform expression in human tissues showed both widespread and differential patterns. CD44 was primarily expressed by epithelial cells. Most epithelia expressed exon v9, but fewer expressed exons v6 or v4. The highest isoform levels occurred in progenitor/stem cells, especially basal cells of stratified squamous and glandular epithelia (33, 34). CD44 isoforms also displayed leukocyte-specific variation. CD44-v9 was expressed minimally in leukocytes, while v6 and v4 were almost undetectable. However, CD44-v9 and v6 were transiently upregulated on activated T cells following mitogen stimulation, and myelomonocytic cells after TNFα/IFNγ exposure. These findings demonstrate that CD44 isoform regulation is both tissue- and cell type-dependent. Differential profiles likely impact respective roles in epithelial homeostasis, tissue repair, and leukocyte immune functions (34, 35). Another study also showed that some epithelial cell lines can preferentially downregulate CIM4-6v and overexpress the CD44v isoform, which easily reaches lymph nodes and forms distant metastases. These results have also been confirmed in breast cancer, which will be further investigated (36).

In the field of diagnosis, this receptor's presence in

Table 1. Functional significance of CD44 isoforms in breast cancer.

CD44 Isoform	Expression in Breast Cancer	Functional Role	Clinical Implication
CD44s (Standard Isoform)	Expressed in various tissues, including cancer cells	Maintains cancer stem cell properties, promotes survival and migration	Potential biomarker for aggressive breast cancer; associated with chemotherapy resistance
CD44v3	Found in highly metastatic breast cancer subtypes	Enhances tumor cell adhesion, regulates growth factor signaling	Target for monoclonal antibody therapies
CD44v5	Upregulated in invasive breast cancer	Promotes cell motility and epithelial-mesenchymal transition (EMT)	Correlates with poor prognosis; potential target for anti-metastatic drugs
CD44v6	Highly expressed in triple-negative breast cancer (TNBC)	Facilitates tumor invasion and angiogenesis	Potential therapeutic target; linked to radioresistance
CD44v8-10	Associated with tumor proliferation and immune evasion	Enhances resistance to oxidative stress and apoptosis	Possible target for immunotherapy and chemotherapy sensitization

various cell types like leukocytes, fibroblasts, epithelial cells, mesoderm, and neuroectoderm aids in cancer diagnosis, since it is naturally present in various stem cells that are also prevalent in cancers (37). To detect a particular type of cancer using the CD44 biomarker, a distinguishing factor from other cancer types is required. This is where the significance of CD44 isoforms emerges. Each isoform is tissue-specific; hence, the evaluation of CD44 cancer is conducted separately for each tissue. Consequently, CD44v can serve as a marker for diagnosing and pre-diagnosing different cancers, such as breast cancer (15). In one study, quantitative RT-PCR and exon-spanning assays were used to examine the expression of alternatively spliced CD44 isoforms and total CD44 levels in 187 breast tumor samples and 13 cell lines. The study also evaluated protein expression of the cancer stem cell marker ALDH1 using IHC in tissue microarrays. Distinct CD44 isoform expression patterns were observed among breast cancer cell lines, and mammosphere development induced changes in this profile. Tumors with robust ALDH1 staining showed increased CD44s and heightened expression of CD44v2–v10, containing all variable exons. CD44 isoforms also correlated with breast cancer subtypes defined by HER2, ER, and PgR status. This implicates the involvement of specific CD44 variants depending on tumor phenotype and suggests that CD44 isoform assessment could serve as a useful diagnostic biomarker (38). In a different investigation, the levels of soluble sCD44v5 and sCD44v6 isoforms were ascertained using a sensitive ELISA technique. Preoperative serum specimens from 82 patients and 67 healthy blood donors of matched ages were evaluated. These findings were compared with clinical and pathological parameters such as tumor size, grade, lymph node metastasis, etc. The investigation found a strong correlation between elevated sCD44v6 levels and lymph node metastasis in breast cancer tumors, indicating sCD44v6 could serve as a diagnostic indicator of cancer tumor progression stages, particularly metastasis (39).

In another study, Gheybi et al. (40) developed a recombinant protein incorporating the variable component of the CD44 (CD44v) extracellular domain for utilization in the clinical diagnosis of breast cancer. A comprehensive analysis identified a total of 100 amino acid residues of CD44v, with structural characterization conducted utilizing bioinformatics methodologies. The genetic construct was subsequently ligated into the PET28a vector and transformed into E. coli BL21(DE3). A fusion protein of approximately 12 kDa was successfully purified through Ni-NTA affinity metal chromatography. The recombinant CD44v was subjected to rigorous examination via Western blotting, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (IHC) assays. The results indicated that the conformation of rCD44v was stable and that its antigenic domain remained accessible. Confirmation of recombinant CD44v was achieved through Western blot analysis, and the presence of antibodies targeting the recombinant CD44v protein in the serum of patients was identified through ELISA. Their findings illustrated a correlation between serum levels of CD44v and the incidence of breast cancer. In conclusion, the assessment of anti-CD44v antibodies in conjunction with rCD44v may serve as a valuable diagnostic tool for the early identification of breast cancer, potentially resulting in improved patient outcomes.

Monoclonal Antibodies and Targeted Therapies for CD44v in Breast Cancer

Perhaps the efficacy of CD44v research can be summarized in the production of monoclonal antibodies. CD44 exists in both standard and variant isoforms generated through alternative splicing. The standard CD44 splice variant (CD44s) contains fixed exons 1–5 and 16–20. Variable exons v1–v10 can combine with the fixed regions to generate different isoforms. One such variant, CD44R1, contains an insertion encoded by exons v8 (exon 13), v9 (exon 14), and v10 (exon 15) (41). CD44R1 expression is elevated in various human epithelial cancers at both the mRNA and protein

levels. For example, CD44R1 mRNA is overexpressed in colon, bladder, lung, laryngeal, and breast cancers. Immunohistochemical analysis also revealed higher CD44R1 protein levels in lung cancer pleural tissues compared to adjacent normal tissues. In this regard, it is possible to increase the efficiency of treatment by producing specific antibodies for each receptor (42). Three anti-CD44 monoclonal antibodies and two anti-CD44v6 monoclonal antibodies were tested for their impact on breast cancer cell lines that expressed varying amounts of CD44s and CD44v6. The antibodies were used to examine the roles of CD44 and CD44v6 in cell adhesion, motility, and invasion. The results showed that anti-CD44 antibodies inhibited adhesion, motility, and invasion of breast cancer cells. In contrast, anti-CD44v6 antibodies only blocked cell motility. This suggests CD44s mediate adhesion, motility, and invasion through interaction with hyaluronan. However, CD44v6 appears to be specifically involved in regulating motility alone (41). In another study, monoclonal antibodies (mAbs) targeting CD44 and CD44v3–10 were generated by immunizing Chinese hamster ovary (CHO)-K1 cells engineered to overexpress CD44v3–10 (CHO/CD44v3–10 cells). The mAbs were then evaluated for their ability to detect CD44v3–10 expression in different cancer cell lines. Specifically, flow cytometry analysis found that the C44Mab-6 antibody could effectively recognize CD44v3 on COLO205 and HSC-3 cells, which are known to upregulate this splice variant. These findings indicate that C44Mab-6 shows promise as a detection reagent for CD44v3 expression in cancers. As CD44v3–10 overexpression has been linked to tumor progression in some studies, the development of tools like this CD44v3-specific monoclonal could aid diagnosis and enable the exploration of therapeutic approaches targeting CD44 variants in the clinic (43).

In a separate study, peptide immunization generated a novel anti-CD44v4 monoclonal antibody called C44Mab-108, suitable for flow cytometry, western blotting, and immunohistochemistry applications (Figure 2). The epitope recognized by C44Mab-108 was elucidated using ELISA with alanine-substituted peptides from the CD44v3–10 amino acid region 271–290. Fluorescence-based assays showed that C44Mab-108 failed to bind peptides with substitutions at D280A and W281A positions, while these peptides did not inhibit C44Mab-108 detection of CD44v3–10 expression. Together, these results precisely mapped the critical binding epitope of C44Mab-108 to encompass the aspartic acid at position 280 and tryptophan at position 281 within the CD44v3–10 sequence. This study demonstrates the value of epitope mapping approaches in developing monoclonal tools with defined targets, which can enable more selective detection and interrogation of specific CD44 isoforms. Notably, all these investigations share a common theme of antibody production centered on diverse isoforms of CD44, underscoring the importance of exploring this subject further (44). In addition to the aforementioned, the use of nanotechnology to enhance the effectiveness of CD44-based therapies has yielded promising results. Gheybi et al. (45) investigated the immunogenic properties of synthesized nanoparticles containing a novel recombinant CD44v (rCD44v) protein within a murine model. The CD44 gene was successfully expressed in E. coli BL21(DE3) utilizing the pET28a-CD44 vector. The expressed rCD44v protein underwent purification, was encapsulated within chitosan nanoparticles, and subsequently administered to BALB/c mice. ELISA was employed to assess the immunoglobulin levels in the immunized subjects. In the challenge experiment, 2×10^6 4T1-CD44 tumor cells were introduced subcutaneously into the mice, followed by measurements of tumor size, necrosis, and metastases. Ultimately, assays for cell proliferation, cytokine production, and neutralization of the mouse anti-rCD44v response against the human breast cancer cell line were conducted. The findings indicated that the recombinant CD44v encapsulated within chitosan nanoparticles enhanced immunological responses attributable to the adjuvant properties of the chitosan nanoparticles. In the immunized murine subjects, titers of IgG and IgA were significantly elevated. Tumor proliferation in both the injection and nano-injection test groups exhibited a statistically significant reduction in comparison to the control groups ($P < 0.05$). A substantial increase in the quantity of splenocytes producing IFNγ and IL-17 was observed in the immunized mice receiving rCD44v ($P < 0.05$). Furthermore, a reduced size of lung metastases was noted when compared to the control groups of mice.

Effective isoforms in breast cancer

CD44 Splice Variants and Their Prognostic Value in Breast Cancer

Years ago, a study was conducted to analyze CD44 splice variant epitopes in human breast cancer for their prognostic potential. Tissues from 91 patients were examined for CD44 variants (v5, v6, v7, v7–v8, and v8–v10) through immunohistochemical staining. The study revealed that standard CD44 antigen expression was found in 54.9% of the patients. Different CD44 epitopes showed varying expression levels: v5 (54.9%), v6 (54.9%), v7 (0%), v7–v8 (34.1%), and v8–v10 (0%). Patients positive for exon v7–v8 differed significantly in tumor size, lymph node status, and degree of lymphatic infiltration compared to those negative for this variant ($p < 0.01$). Thus, isoforms v6 and v5 emerge as crucial in this context (34). In a study on female breast cancer, the expression frequency of the CD44 marker and its variants CD44v3 and CD44v5 was examined. Out of 75 cases, 44.2% displayed a strong membrane reaction to the CD44 marker. CD44v3 and CD44v5 were detected in 21.3% and 66.75% of cases, respectively. The presence of CD44v3 correlated significantly with ER expression,

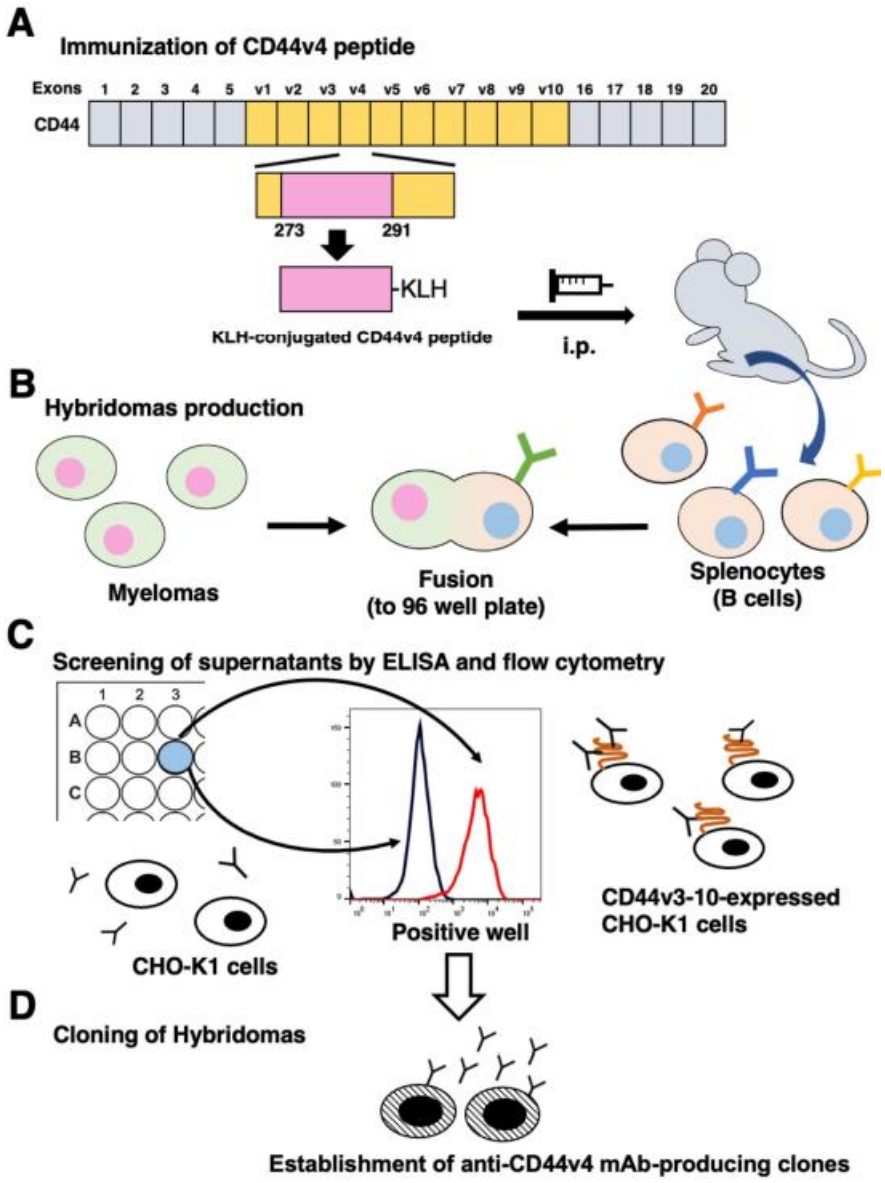

Figure 2. An outline of the production process for anti-CD44v4 mAbs. (A) The CD44v4 peptide conjugated with keyhole limpet hemocyanin (KLH) was administered intraperitoneally to the mice. (B) Hybridomas were created by fusing P3U1 cells with spleen cells. (C) The supernatants were then subjected to flow cytometry utilizing CHO/CD44v3-10 and CHO-K1 cells after being screened using the CD44v4 peptide in an enzyme-linked immunosorbent assay (ELISA). It was found that both parental CHO-K1-non-reactive and CHO/CD44v3-10-reactive supernatants tested positive for CD44v3-10. (D) Limiting dilution was used to create single clones. Finally, a clone C44Mab-108 (IgG1, kappa) was created. Reprinted from (44).

absence of protein, and T cell percentage in lymphocytes. Additionally, the CD44v3 reaction showed a notable association with lymph node metastasis (15, 46).

A separate study investigated CD44 expression in 75 breast tissue samples with benign, pre-malignant, and malignant lesions using immunohistochemistry. In benign lesions, CD44v6 was detected in 20–30% of ductal epithelial cells, while CD44s, CD44v3, and CD44 were absent. CD44v3 and CD44v6 were overexpressed in localized and invasive carcinomas. However, CD44 levels in carcinoma cells did not correlate with tumor type or differentiation. Additionally, CD44 and HA expression levels were not closely associated with benign or malignant breast lesions. These findings demonstrate isoform-specific patterns of CD44 splicing in breast tumorigenesis, with CD44v3 and v6 upregulation linked to malignant progression. However, total CD44 levels may have limited utility as a breast cancer biomarker, according to the differential and non-correlative expression profiles observed (47).

CD44 Isoform Interactions and Their Role in Cancer Metastasis

In a study, CD44 isoforms influencing TrkA/CD44 complex formation were identified via proximity ligation methods. Molecular determinants of this interaction were investigated through molecular modeling, isolating the involved amino acids and confirming their roles via mutation. A CD44v3 mimic peptide was synthesized to disrupt the TrkA/CD44v3 interaction, and its impact on the growth, migration, and invasion of xenograft triple-negative breast cancer cells was assessed. The study also explored the correlation between TrkA/CD44v3 expression in tumors and histo-prognostic parameters. Results indicated that the CD44v3 isoform, not v6, binds to TrkA upon NGF stimulation. The last 10 amino acids of exon v3 and TrkA residue H112 are crucial for CD44v3–TrkA association. Functionally, the CD44v3 mimetic peptide hindered NGF-induced RhoA activation, clonogenesis, and migration/invasion of breast cancer cells in vitro. In a xenograft mouse model, it reduced tumor growth and metastasis. TrkA/CD44v3 was exclusively found in cancer cells, not in adjacent normal tissues. These findings suggest that targeting the CD44v3/TrkA interaction could be a promising therapy for triple-negative breast cancer (48). Tumor necrosis factor-α (TNF-α) is overexpressed in the tumor microenvironment and can influence CD44 expression in certain cancers. However, its role in breast cancer is not well defined. Recent studies investigated the impact of TNF-α on breast cancer cell lines MCF-7 and MDA-MB-231. TNF-α was found to decrease CD44 expression while upregulating CD44v3 and CD44v6 in MCF-7 cells, mediated through the JNK signaling pathway. In the more invasive MDA-MB-231 cells, TNF-α regulated total CD44 along with CD44v3 and v6 isoforms via the p38 pathway. These findings suggest that TNF-α modulates CD44 splicing in a cell type-specific manner. By disturbing the balance of different CD44 isoforms via distinct MAPK mechanisms, TNF-α could potentially promote breast cancer progression by altering cell survival, migration, and other malignant phenotypes regulated by CD44 variants (49).

Clinical Significance of CD44 Isoforms in Metastasis and Treatment Response

Numerous studies have investigated the clinical significance and prognostic value of CD44v5 and CD44v6 expression in primary breast cancers, though results have often been conflicting. One such study analyzed serum levels of soluble CD44v5 (sCD44v5) and CD44v6 (sCD44v6) in breast cancer patients to evaluate correlations with metastatic disease status, primary tumor expression of these variants, and the location of metastases. Serum samples were collected from two groups—patients who had developed metastases in different organs, and a second group with single-organ metastases only. Control samples included patients who remained non-metastatic after surgery, as well as serum from healthy volunteers. Immunohistochemical analysis was performed to examine the expression of membrane-bound CD44v5 and CD44v6 in primary breast tumors from patients with metastases to different organs. Serum levels of sCD44v5 and sCD44v6 were then measured using ELISA assays. When metastases were present, mean serum levels of both sCD44v5 and sCD44v6 were found to be significantly higher compared to levels measured one month after tumor resection in non-metastatic patients. Six of the nineteen patients tested had elevated sCD44v5 above the cutoff value of 85 ng/mL, correlating with increased CD44v5 expression in their primary cancers. Similarly, six of twenty patients showed increased sCD44v6 above 275 ng/mL, associated with higher primary tumor CD44v6 levels. Among 57 patients with single-organ metastases, statistical analysis revealed that serum sCD44v6—but not sCD44v5—was specifically associated with liver or bone metastases. Immunoblot analysis identified two distinct CD44v6 proteins at approximately 120 kDa and 170 kDa in patient sera. Together, these findings indicate that serum concentrations of sCD44v5 and sCD44v6 reflect the expression of their respective variants in primary breast tumors and may provide insight into metastatic burden and organ-preferential spread (50, 51). Other studies have investigated the expression of activation markers on breast epithelium surrounding silicone gel-filled breast implants (SGBI) to gain insights into local tissue responses. Using an immunohistochemical approach, 12 tissue samples from women with implants were examined for CD44v6, ICAM-1, HLA-DR, and HLA-DQ expression and compared to 6 control tissues from patients with mild breast hyperplasia. CD44v6 was detected on ductal epithelium in all implant samples. HLA-DR expression was also observed in all implant tissues, while HLA-DQ was positive in three samples and ICAM-1 in eight cases. In contrast, the control hyperplasia tissues showed no expression of CD44v6, HLA-DQ, or ICAM-1, and only weak HLA-DR staining in two samples. These findings suggest that under certain conditions—such as proximity to breast implants—CD44v6 and other activation markers can be abnormally expressed by typically quiescent breast ductal epithelium, indicating a stimulated state. Elucidating molecular alterations surrounding implants may provide insights into local tissue responses to foreign material (52, 53).

CD44v7–v8 antigen expression in breast cancer is an important independent prognostic factor and is strongly associated with lymph node invasion status. Fourteen of 31 patients positive for CD44v7–v8 experienced relapse, with recurrence manifesting as lymphatic metastasis in 10 of these 14 cases. Breast cancer cells expressing the CD44v7–v8 antigen show high affinity for lymph nodes and lymphatic vessels and are prone to metastasize to distant lymph nodes

even in the early stages of the disease (54, 55). In the field of metastasis, several studies have investigated the role of CD44v7–v8 in cancer progression. Osteopontin (OPN), a glycosylated phosphoprotein, enhances cell migration and chemotaxis, potentially promoting tumor progression. OPN boosts the metastatic potential of transformed cells, particularly in breast cancer, through interactions with integrins and CD44 receptors (56, 57). One study proposed that OPN influences specific CD44 isoforms to promote breast cancer cell motility. Using the 21NT breast cancer cell line, researchers assessed CD44 expression under elevated OPN levels. They observed increased mRNA levels for certain CD44 isoforms but no changes in CD44v6, v8, v9, or v10 mRNA. While CD44s, v6, and v9 proteins were abundant on the cell surface, only CD44s and v6 showed significant increases in total cellular protein. These findings indicate OPN's regulation of CD44 expression at both transcriptional and post-transcriptional levels. To functionally validate this regulation, OPN-mediated cell migration was shown to be reduced by exposure to function-blocking antibodies targeting CD44v6 and CD44v9 (56).

The impact of CD44v7–v8 on neoadjuvant chemotherapy (NAC) is particularly relevant in triple-negative breast cancer (TNBC), where NAC is the standard treatment. Patients with residual disease after NAC have worse survival compared to those who achieve a pathologic complete response (pCR), which remains the only definitive prognostic factor (58). A study involving 48 TNBC patients treated with NAC evaluated CD44v9 (a stemness marker), vimentin (an EMT marker), BRCA1, and baseline phenotypes via immunohistochemistry. Of the 48 patients, 14 achieved pCR, with high baseline and nuclear phenotypes significantly associated with this outcome. No significant correlation was found between pCR rate and expression of CD44v9, vimentin, or BRCA1. Achieving pCR was associated with improved distant metastasis-free survival (DMFS), while high CD44v9 expression correlated with shorter DMFS. In non-pCR patients, high-grade residual tumors, poor pathological response, and elevated CD44v9 expression in pretreatment samples were linked to worse DMFS. Furthermore, high-grade residual tumors showed significant association with pre-treatment CD44v9 overexpression (58).

Conclusion

Breast cancer stem cells exhibit a significant increase in CD44 biomarker expression, which is associated with drug resistance, increased radiation therapy resistance, and a higher likelihood of metastasis, invasion, proliferation, and overall tumor growth. Consequently, recent research has focused on targeting this biomarker and the cancer stem cells that express it. To effectively utilize CD44 as a biomarker in breast cancer diagnosis and treatment, it is crucial to understand its various isoforms, each with unique amino acid sequences and chemical properties that influence the receptor's function. These isoform characteristics can significantly impact therapeutic outcomes. Currently, innovative therapeutic approaches are being explored for breast cancer treatment, including recombinant monoclonal antibodies and artificial ligands that specifically bind to CD44 as neutralizing agents. This research highlights the importance of targeting CD44 and its isoforms to develop more effective cancer therapies.

Conflict of interests

The authors declare no conflict of interest.

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Author's Contributions

Elaheh Gheybi: Writing – original draft, Conceptualization. **Pejman Hosseinzadeh:** Writing – original draft, Conceptualization. **Vahid Tayebi-Khorrami:** Writing – review & editing. **Mehdi Rostami:** Writing – review & editing. **Mohammad Soukhtanloo:** Writing – review & editing, Supervision, Conceptualization.

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