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A NEW CD44v3-CONTAINING ISOFORM IS INVOLVED IN TUMOR CELL GROWTH AND MIGRATION DURING HUMAN BREAST CARCINOMA PROGRESSION

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1. ABSTRACT

CD44 isoforms belong to a family of cell adhesion molecules expressed on the cell surface of many tumor cells during human breast cancer progression. In this study we have analyzed the expression of CD44v3-containing isoforms [containing heparan sulfate addition sites for growth factor binding] in primary breast tumors, axillary nodal metastases and normal breast tissue. Using reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blot, cloning, nucleotide sequencing and RT-in situ-PCR analyses, we have found that at least two CD44v3-containing isoforms, including one new species of CD44v2,Δv3-10 (Δv3 defined as a v3 exon lacking the first 24 base pairs) and another previously reported CD44v3,8-10 are preferentially expressed in human primary breast tumor and axillary nodal metastases but not in normal breast tissues. These finding suggest that these CD44v3-containing isoforms are closely associated with breast cancer metastasis.

Furthermore, we have established a stable transfection of CD44v2,Δv3-10 cDNA into non-metastatic human breast tumor cells (MCF-7) which contain endogenous CD44E isoform. Our results indicate that expression of CD44v2,Δv3-10 in MCF-7 cells promotes tumor cells to undergo rapid cell growth and active cell migration. Treatments of MCF-7 transfectants expressing CD44v2,Δv3-10 with various agents such as anti-CD44v_3 antibody, cytochalasin D (a microfilament disrupting agent known to prevent actin polymerization) and W-7 (a calmodulin antagonist) but not colchicine (a microtubule inhibitor), cause a significant inhibition of tumor cell migration. These findings suggest that CD44v2,Δv3-10 (related to human metastatic breast cancers) and associated microfilament components play an important role in the regulation of breast tumor cell behaviors required for the progression of human breast carcinomas.

2. INTRODUCTION

The transmembrane glycoprotein CD44 isoforms are all major hyaluronic acid (HA) cell surface receptors that exist on many cell types, including macrophages, lymphocytes, fibroblasts and epithelial cells (1-5). Due to its widespread occurrence and its role in signal transduction, CD44 isoforms have been implicated in the regulation of cell growth and activation as well as cell-cell and cell-extracellular matrix interactions (1-7).

One of the distinct features of CD44 isoforms is the enormous heterogeneity in the molecular masses of these proteins. It is now known that all CD44 isoforms are encoded by a single gene which contains 19 exons (8). Out of the 19 exons, 12 exons can be alternatively spliced (8). Most often, the alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons [v1-v10 (exon 6-exon 14) in human cells] within the membrane proximal region of the extracellular domain (8). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N-and O-glycosylations and glycosaminoglycan (GAG) additions (9-12). In particular, CD44v3-containing isoforms have a heparin sulfate addition at the membrane-proximal extracellular domain of the molecule that confers the ability to bind heparin sulfate-binding growth factors (9,10).

Cell surface expression of CD44v isoforms changes profoundly during tumor metastasis, particularly during the progression of various carcinomas including breast carcinomas (13-16). In fact, CD44v isoform expression has been used as an indicator of metastasis. In this study we have identified at least two CD44v3-containing isoforms, including one new species of CD44v2,Δv3-10 (Δv3 defined as a v3 exon lacking the first 24 base pairs) and another previously reported CD44v3,8-10 which appear to be in a close association with human primary breast tumor and axillary nodal metastases but not in normal breast tissues.

In addition, we have cloned CD44v2,Δv3-10 cDNA into a pRc/CMV vector and transfected this cDNA into non-metastatic human breast tumor cells (MCF-7) containing endogenous CD44E isoform. We have determined that the expression of CD44v2,Δv3-10 in MCF-7 promotes tumor cell growth and migration. These results suggest that the expression of CD44v2,Δv3-10 is involved in the regulation of tumor cell behavior required for the progression of human breast carcinomas.

3. MATERIALS AND METHODS

3.1. Human Breast Carcinoma Samples

Human breast carcinoma samples (e.g. breast carcinomas, axillary nodal metastases and normal breast tissues) were obtained from the tissue bank core facility in the Sylvester Comprehensive Cancer Center at the University of Miami Medical School. These samples were collected after radical mastectomy and characterized pathologically in terms of their stage in malignancy. Specifically, breast tissue samples, 0.5-1.0 cm in diameter, were obtained from fresh surgical resection specimens obtained from patients with breast tumors. The samples were snap-frozen in liquid nitrogen within 10 min of arrival in the pathological specimen reception area and were kept frozen until use. Portions of any lymph node metastasis in the resected tissue have also been collected. Normal breast tissue and lymph node were obtained from the periphery of specimens surgically resected for treatment of cancer, and from other specimens removed for non-neoplastic conditions such as fibrocystic disease of the breast.

3.2. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and One-Step Cloning

Total RNA was extracted from 200-400 mg of frozen human breast tumor tissue, axillary nodal metastatic tissue, and normal human breast tissue using the acid guanidinium thiocyanate-phenol-chloroform technique of Chomczynski and Sacchi (17). Approximately 3μg of total RNA was used with an oligo(dT) primer in a reverse transcriptase system (Promega, Madison, WI) to synthesize complementary DNA(cDNA) at 42°C for 1h, using AMV(avian myeloblastosis virus) reverse transcriptase. After synthesis of the first strand, polymerase chain reaction (PCR) of the CD44cDNA was done by initial melting of the RNA/cDNA at 94°C for 4 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and polymerization at 72°C for 1 minute. The PCR primers were designed initially to look at and amplify the region involved with alternative splicing. Specifically the 2 primers were CD44 exon 4 (exon 4: 5'-TACATCAGTCACAGACCTGC-3') and CD44 exon 14 (exon 14: 5'-CTGCAGTAACTCCAAAGGAC-3'). Analysis of the presence of v3-containing isoforms was facilitated by designing PCR primers within exon 5 and v3 (exon 7). Specifically the primers were CD44 exon 5 (exon 5: 5'-GCACTTCAGGAGGTTACATC-3') and CD44 v3(exon 7) (exon 7: 5'-CTGAGGTGTCTGTCTCTTTC-3'). Analysis for the presence of v2v3-containing isoforms was done by using the previously designed exon 5 left primer and designing a v2v3 specific right junction primer, specifically CD44 v2/v3 (exon 6/7) (exon 6:5'-GAAGACGTACCAGCCATTTG-3'). The PCR products were one-step cloned using TA-cloning kit (Invitrogen, San Diego, CA) and sequenced by dideoxy sequencing method.

3.3. Southern Blot Analysis

The PCR products were separated on a 2% agarose gel, blotted on to the nitrocellulose filter and hybridized to [^{32}P]dCTP-labeled CD44 cDNA probes. In a control experiment, RT-PCR was done in the absence of AMV reverse transcriptase or in the absence of template in the PCR reaction. No amplification products were found by Southern blot in these negative control samples.

3.4. Immunohistochemistry and RT *in situ* PCR

The breast tissue specimen was fixed in formalin, embedded in paraffin and sectioned at 4.0 μm thickness. The tissue section was subjected to immunofluorescence staining using rabbit anti-CD44v3 antibody followed by rhodamine-conjugated goat anti-rabbit IgG. For RT *in situ* PCR analysis of CD44v3-containing transcript in breast tissues, a protocol by Nuovo[18] was used with following modifications. In the PCR mixture we used the direct incorporation of fluorescein (FITC)-digoxigenin-11-dUTP and the CD44v3 (exon 7) specific primers (as described above), and 20 PCR cycles were carried out at 50^o C for 2 min and 94^o C for 1 min. The fluorescent signals were then analyzed by a laser scanning Confocal microscope (MultiProbe 2001 Invert CLSM System, Molecular Dynamics) using a 63 X-oil immersion and an imaging processing device. Images were photographed with Kodak TMAX100 Film.

3.5. Cell Lines

The MCF-7 cell line was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % fetal calf serum, L-glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml) (GIBCO BRL, Gaithersburg, MD). The cells were maintained in a humidified incubator under a 5 % CO_2 in air atmosphere at 37^{o}C.

3.6. Stable Transfection

MCF-7 cells were transfected with pcDNA3 plasmid (Invitrogen, San Diego, CA) containing the human CD44v2,Δv3-10 cDNA using the LIPOFECTAMINE reagent (Gibco, Grand Island, NY) according to the manufacturer's protocol. Control transfections consisted of either no DNA or pcDNA3 vector DNA. Stable cell lines were established and maintained in Minimum Essential Medium (Gibco) supplemented with 10% FBS, and containing 500 μg/ml G418. Three days after transfection, cells were split 1:10 in complete medium containing G418 (GIBCO BRL) at a concentration of 750 μg/ml. Individual colonies were isolated after two weeks; and stable cell lines were established and maintained in the complete medium containing 400 μg/ml G418. The growth rate of transfected cells with CD44v2,Δv3-10 appears to be similar to that in the parental cells/vector-transfected control cells.

3.7. Analysis of CD44 Expression At the Protein Level

Transfected and parental MCF-7 cells were analyzed by polyacrylamide gel (7.5%) electrophoresis followed by immunoblotting with monoclonal rat anti-CD44 antibody (Clone:020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., Miami, FL.) which recognizes a common determinant of the CD44 class of glycoproteins, including CD44s and other variant isoforms (14). The blots were then incubated with peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h. After an addition of peroxidase substrate (Pierce Co.), they were then developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

3.8. *In vitro* Cell Migration Assays

Twenty-four transwell units were used for monitoring *in vitro* cell migration as described previously (19). Specifically, the 8μm porosity polycarbonate filters were used for the cell migration assay (19). MCF-7 cells [e.g. parental cells (untransfected), control cells (transfected with

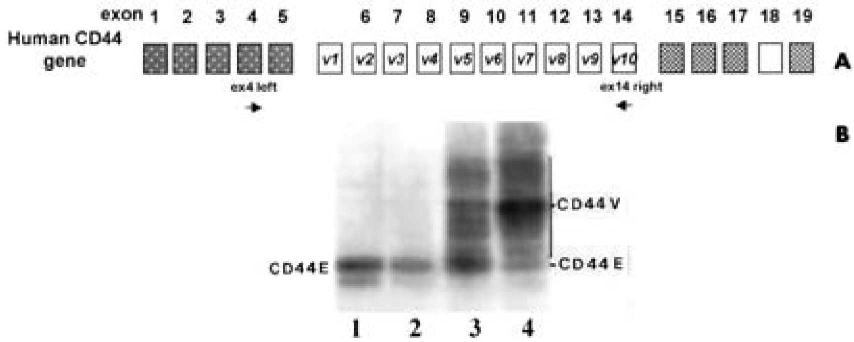

Figure 1: A representative RT/PCR and Southern blot analyses of CD44 isoforms using RNAs isolated from human breast tissues. Total RNA isolated from breast tissues were reverse-transcribed and subjected to PCR primer pairs (exon 4 and exon 14) as described in figure 1A. Subsequently, RT-PCR products were analyzed by Southern blot hybridization as shown in figure 1B (lane 1 and 2: normal breast tissues; lane 3: primary breast tumor tissues; lane 4: axillary nodal metastatic breast tumor tissues). [CD44E, CD44 epithelial form; CD44v, CD44 variant isoforms].

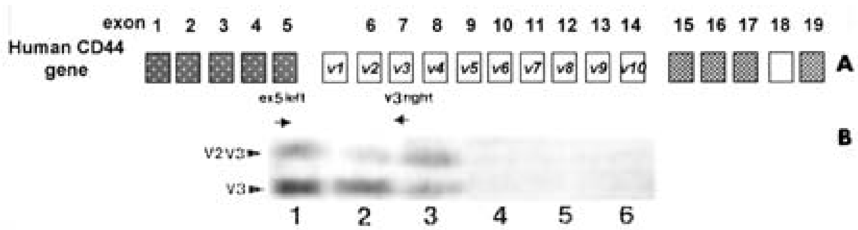

Figure 2: RT/PCR and Southern blot analyses of v3-containing CD44 isoforms using RNAs isolated from human breast tissues. Total RNA isolated from primary breast tumor tissues were reverse-transcribed and subjected to PCR using PCR primer pairs [exon 5 and v3 (exon 7)] as described in Figure 2A. Subsequently, RT-PCR products were analyzed by Southern blot hybridization as described in the Materials and Methods. B: Southern blot reveals the presence of v3-containing CD44 transcripts (V3) as well as a larger band that represents insertion of the v2v3 exon. These two PCR products (v3 and v2v3) were then cloned into the pCRII vector (Invitrogen Corp., San Diego, CA) and sequenced as described in the Materials and Methods. The nucleotide sequences confirm that these species belong to v3 and v2v3. (lane 1: primary breast tumor tissues; lane 2 and 3: axillary nodal metastatic breast tumor tissues; lane 4 and 5: normal breast tissues; lane 6: RT-PCR was carried out in the absence of reverse transcriptase using metastatic breast tumor tissues as a negative control).

vector only) or transfectants (containing CD44v2,Δv3-10 cDNA)] [≈1 x 10^{-4} cells/well in phosphate buffered saline (PBS), pH 7.2] were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DMEM supplemented by 10% fetal bovine serum was placed in the lower chamber of the transwell unit. After 18h incubation at 37^{o}C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously[19]. Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically by Student's t test and statistical significance was set at $p<0.01$.

3.9. *In vitro* Cell Growth Assays

MCF-7 cells [e.g. parental cells (untransfected), control cells (transfected with vector only) or transfectants (containing CD44v2,Δv3-10 cDNA)] (5 x 10^3 cells/well) were plated in 96-well culture plates in 0.2ml of Dulbecco's modified Eagle's medium/F12 medium supplement (GIBCO, Grand Island, NY) containing either 0.5% fetal bovine serum or no serum for various time (e.g. 24h, 48h and 72h) at 37^{o}C in 5%CO_2/95% air. In each experiment, a total of 5 plates (10 wells/cell line) was used. Experiments were repeated three times. The *in vitro* growth of these cells were analyzed by measuring increases in cell number using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (CellTiter 96^R non-radioactive cell proliferation assay according to the procedures provided by Promega, Co.). Subsequently, viable cell-mediated reaction products were recorded by a Molecular Devices (Spectra Max 250) ELISA reader at a wavelength of 570nm.

4. RESULTS

4.1. Identification of CD44 Variant Transcripts Expressed in Human Breast Carcinoma Tissues By RT-PCR, Southern Blot Analyses, cDNA Cloning and Nucleotide Sequencing

In this study we have used the RT-PCR technique and specific primer pairs (exon 4 and exon 14) (Figure 1A) to analyze the transcript expression of various CD44 isoforms in both normal and breast carcinoma tissues. Our results indicate that normal human breast tissues contain primarily one major band of CD44-related PCR product (Figure 1B, lane 1 and lane 2). This PCR product was subsequently "one-step cloned" into the pCR^{TM} vector from Invitrogen Corporation and sequenced. Our nucleotide sequence data indicate that it represents the CD44 epithelial (CD44E) form (Figure 1B, lane 1 and lane 2). Interestingly, the CD44E form is also expressed in primary breast tumor tissues (Figure 1B, lane 3) and metastatic breast carcinomas (Figure 1B, lane 4). Furthermore, multiple species of the CD44-related gene products [i.e. CD44 variant isoforms (CD44v) have been found in primary breast tumor tissues (Figure 1B, lane 3) and axillary nodal metastases tissues invaded by breast carcinoma cells (Figure 1B, lane 4). These findings are consistent with previous findings showing that several large size CD44 variant isoforms exist in breast carcinoma cells (13-16).

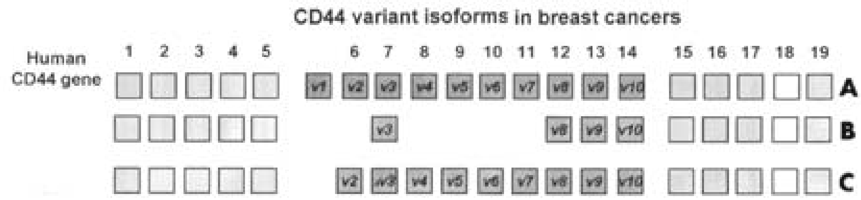

Figure 3: Exon map of CD44 in human (A) and schematic illustration of the CD44$v_{3,8\text{-}10}$ isoform (B) and CD44v2,Δv3-10 isoform (C) detected in primary breast tumor tissues and axillary lymph nodal metastases as described in Figs. 1 and 2.

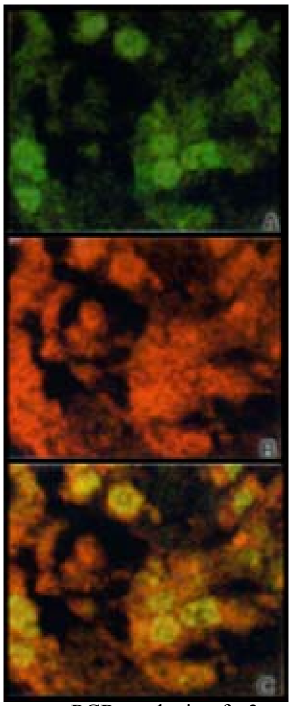

Figure 4: RT *in situ* PCR analysis of v3-containing CD44 transcripts and proteins in breast carcinomas tissues. Breast carcinomas tissue specimen was fixed with formalin, paraffin embedded, sectioned (4 µm) and processed for RT *in situ* PCR and immunofluorescence staining as described in the Materials and Methods. A: Detection of CD44v3-specific transcripts by RT *in situ* PCR using a v3-specific primer pair and FITC-digoxigenin-11-dUTP as described in the Materials and Methods. B: Immunofluorescence staining of CD44v3-containing proteins using rabbit anti-CD44v3 antibody followed by rhodamine-conjugated goat anti-rabbit IgG. C: Colocalization of CD44v3-specific transcripts (A) and proteins (B) by combining two images (A and B) together.

The v3 (or exon 7) insertion of CD44v3 isoforms has been shown to contain heparin sulfate addition sites required for the binding of a wide range of heparin binding growth factors, cytokines, and chemokines for promoting cell growth (9) or tumor cell migration (20). These observations have prompted us to examine the possible expression of CD44v3-containing isoforms in these breast carcinoma tissue specimens. To detect the expression of CD44v3-containing transcript(s) at the RNA level of various breast carcinoma tissues, total RNA from these materials was extracted and analyzed by RT-PCR using exon specific primers. Using a PCR primer pair to amplify between exon 5 and v3 (exon 7) (Figure 2A) by RT-PCR followed by Southern blot analyses, we have detected the presence of two v3-containing species in the primary breast tumor tissues (Figure 2B, lane 1) and axillary nodal metastases (Figure 2B, lane 2 and 3) but not in normal breast tissues (Figure 2B, lane 4 and 5). These two v3-containing PCR products were then "one-step cloned" into the pCRII vector from Invitrogen Corp. (San Diego, CA) and sequenced. The nucleotide sequences confirm that one band belongs to the v3 exon insertion and the other band (the larger band) represents v2v3 exon insertion (Figure 2B, lane 1-3). We believe that the RT-PCR reaction is specific since no amplified fragment can be detected in samples without any reverse transcriptase (Figure 2B, lane 6).

Furthermore, PCR products of CD44 amplified between exons 4 and 14 detected in both primary breast tumor tissues (Figure 1B, lane 3) and axillary nodal metastases (Figure 1B, lane 4) were then analyzed for these v3 and v2v3-containing isoforms. Using the technique of colony hybridization with a v2v3-specific probe, two unique colonies that contained v3 were detected. Specifically, the results from the nucleotide sequence analysis reveal the presence of two CD44v3-containing isoforms, CD44v3,8-10 (Figure 3B) and CD44v2,Δv3-10 (Δv3 defined as a v3 exon lacking the first 24 base pairs) (Figure 3C).

4.2. Immunofluorescence and RT *in situ* PCR

The expression of CD44v3-containing transcripts and proteins were also analyzed in breast carcinoma tissues by RT *in situ* PCR analysis (Figure 4A) and immunofluorescence staining (Figure 4B). The results from the RT *in situ* PCR using the v3 (exon 7) specific primer pair show the presence of a strong fluorescent signal in the breast carcinomas tissues (Figure 4A). This signal is absent in the negative control samples where the reverse transcriptase was eliminated from the RT reaction mixture (data not shown). The location of CD44v3-containing proteins in breast carcinomas tissues was also established by immunofluorescence staining using anti-CD44v3 antibody (Figure 4B). The fact that both CD44v3-containing RNA transcripts and proteins are colocalized in the same population of breast carcinomas (Figure 4C) suggests that the CD44v3-containing transcripts (e.g. CD44v2,Δv3-10 and CD44v3,8-10) are successfully expressed at the protein level in breast carcinoma tissues.

4.3. Transfection and Expression of CD44v2,Δv3-10 In Human Breast Tumor Cells (MCF-7) Containing CD44

In this study certain CD44 variant (CD44v) isoforms, such as CD44v3,8-10 and CD44v2,Δv3-10 have been found to be preferentially associated with human breast carcinomas cells in both early primary tumors and the advanced stages of axillary nodal metastases (Figs. 1-3). The biochemical characteristics of the CD44v3,8-10 isoform (but not the CD44v2,Δv3-10 isoform) have been described previously by a number of investigators (9,20). Consequently, we have decided to focus on analyzing the structural and functional properties of the new species of CD44v3-containing isoform, CD44v2,Δv3-10. In order to test directly whether the expression of the CD44v2,Δv3-10 isoform is involved in breast tumor cell behavior (e.g. cell

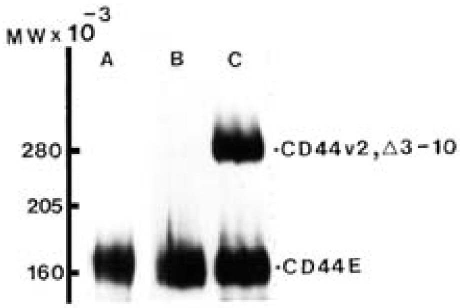

Figure 5: Analysis of CD44v2,Δv3-10 isoform expression in MCF-7 transfectants by monoclonal rat anti-CD44-mediated immunoblot. Lane A: Anti-CD44-mediated immunoblot of the cell lysate obtained from parental (untransfected) MCF-7 cells [revealing the presence of CD44E (≈160 kDa)]. Lane B: Anti-CD44-mediated immunoblot of the cell lysate obtained from the control (vector-transfected) MCF-7 cells [revealing the presence of CD44E (≈160 kDa)]. Lane C: Anti-CD44-mediated immunoblot of the cell lysate obtained from MCF-7 transfectants containing CD44v2,Δv3-10 cDNA [revealing the presence of CD44v2,Δv3-10 (≈280 kDa) plus CD44E (≈160 kDa)].

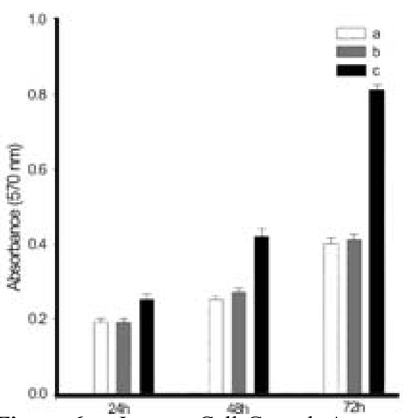

Figure 6: *In vitro* Cell Growth Assays. The *in vitro* cell growth of breast tumor cells [e.g. MCF-7 transfected with CD44v2,Δv3-10 cDNA, parental (untransfected) MCF-7 cells, and the control (vector-transfected) MCF-7 cells (5 x 10^3 cells/well) was measured at various time (e.g. 24h, 48h and 72h) by the MTT assays as described in the Materials and Methods. a: Cell growth of the parental (untransfected) MCF-7 cells.b: Cell growth of the control (vector-transfected) MCF-7 cells. c: Cell growth of MCF-7 cells transfected with CD44v2,Δv3-10 cDNA.

Table 1. *In vitro* Tumor Cell Migration Assays

Treatments	Cell Migration[a] (Cell Number/Field)[b]
Parental Cells (untransfected)	10
Control Cells (transfected with vector only)	15
Transfectants (containing CD44v2,Δv3-10 cDNA)	155

a: The procedures for the *in vitro* cell migration assay were described in the Materials and Methods.
b: The values expressed in this table represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ± 5%.

growth and/or migration), we have established a new, stable transfectant of a breast tumor cell line which is capable of expressing CD44v2,Δv3-10 on its cell surface. Specifically, CD44v2,Δv3-10 cDNA was cloned into a pRc/CMV vector and expressed in non-metastatic human breast tumor cells (MCF-7) containing endogenous CD44E (but not CD44v2,Δv3-10). Using anti-CD44-mediated immunoblotting techniques, we have found that both the parental (untransfected) cells and control cells (transfected with vector only) contain only one large endogenous CD44E isoform (M.W. ≈ 160kDa) on the cell surface (Figure 5A and 5B). In cells transfected with CD44v2,Δv3-10 cDNA, one additional CD44-related polypeptide (M.W. ≈280kDa) is expressed on the cell surface (Figure 5C). Routinely, we selected at least 8 cloned MCF-7 transfectants from each transfection experiment based on their ability to express CD44v2,Δv3-10 (≈280kDa) on the cell surface. Since all 8 cloned transfectants express an identical amount of CD44v2,Δv3-10 (≈280kDa) on the cell surface and display similar biological properties (e.g. cell growth properties and migration capability, etc.), in this study we have decided to show one representative MCF-7 transfectant [expressing CD44v2,Δv3-10 (≈280kDa) (Figure 5C)] to address the effects of CD44v2,Δv3-10 expression on tumor cell behavior(s) as described below.

4.4. Analyses of Tumor Cell Growth and Migration of MCF-7 Transfectants Expressing CD44v2,Δv3-10

The common characteristics of metastatic tumor cells is their ability to undergo rapid cell growth and migration. Using *in vitro* cell growth assays, we have found that the MCF-7 transfectants expressing CD44v2,Δv3-10 (Figure 6c) display significantly increased cell growth compared with the parental (untransfected) cells (Figure 6a) and control cells (transfected with vector only) (Figure 6b). The results from *in vitro* cell migration measurements also indicate that the MCF-7 transfectants expressing CD44v2,Δv3-10 are capable of undergoing a very active cell migration (Table 1). On the contrary, both the parental (untransfected) cells and control cells (transfected with vector only) show relatively low migration activities (Table 1). These findings suggest that CD44v2,Δv3-10 plays an important role in the regulation of breast tumor cell growth and motility.

5. DISCUSSION

CD44 variant isoforms have been shown to be closely involved in the onset of tumor development and metastasis. For example, certain CD44 variant (CD44v) isoforms appear to be expressed at high levels on the surface of tumor cells during tumorigenesis and metastasis (13-16). Specifically, one of the CD44v isoforms, CD44v6, has been correlated with an advanced tumor stage and poor patient survival in non-Hodgkin's lymphoma (21) and colorectal tumors (22). This CD44v6 isoform has also been found to confer metastatic behavior on rat pancreatic cells in a spontaneous metastasis assay (23). In addition, CD44v5 expression has been proposed as an early tumor marker for colorectal carcinoma since it is detectable on dysplastic colon polyps and carcinoma, yet is not present on normal intestinal epithelium (22). Furthermore, increased CD44v7/8 expression has been found during the progression of human cervical carcinoma, showing its presence in almost 100% of tissue samples at the stage of carcinoma in-situ (24). A positive association between the presence of CD44v9 and the progression of human gastric carcinoma have also been demonstrated (25). Recently, CD44v10 is shown to be involved in the onset of breast tumor development and progression (14,28).

In this study we have used the RT-PCR technique and nucleotide sequence analysis to study the expression of CD44 variant isoforms in primary breast tumors, axillary nodal metastases and normal breast tissue. Our data indicate that both primary breast tumor tissues (Figure 1B, lane 3; Figure 2B, lane 1) and metastatic breast carcinomas (Figure 1B, lane 4; Figure 2B, lane 2 and 3) display a high level of CD44 variant isoform expression compared to the very low level seen in normal breast tissue (Figure 1B, lane 1 and lane 2; Figure 2B, lane 4 and 5). These results are consistent with previous findings linking the expression of various CD44 variant isoforms to tumor progression and metastasis in several types of cancers (14,21-28).

As the histologic grade of each of the tumors progresses, the percentage of lesions expressing an associated CD44 variant isoform increases. In particular, the CD44v3-containing isoforms are expressed preferentially on highly malignant breast carcinoma tissue samples. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (14,26). One study indicates that breast tumor expression of the CD44v3 isoform may be used as an accurate predictor of overall survival (e.g. nodal status, tumor size, and grade) (14,27).

When analyzed for the presence of the v3 exon, we have found that primary tumors (Figure 2B, lane 1) and axillary metastases (Figure 2B, lane 2 and 3) contain v3 as well as v2v3 while normal breast tissue does not (Figure 2B, lane 4 and 5). We have also determined that these two CD44v3-containing isoforms represent CD44v2,Δv3-10 (Figure 3C) and CD44v3,8-10 (Figure 3B). The fact that both CD44v3-containing RNA transcripts (Figure 4A) and proteins (Figure 4B) are localized (Figure 4C) in the same population of breast carcinoma cells by RT-in situ-PCR and immunofluorescence staining suggests that these CD44v3-containing isoforms are closely associated with breast carcinoma progression.

The metastatic phenotype of tumor cells is characterized by rapid tumor cell growth and cell motility (29,30). Previously, a number of CD44 isoforms have been implicated in signal transduction, cell activation, tumor cell growth and metastasis (31-39). Most recently, one of the v3-containing CD44 variant isoforms such as $CD44v_{3,8-10}$ is shown to be linked to the cytoskeleton during breast tumor cell motility (20). Using a stable transfection of CD44v2,Δv3-10 cDNA into non-metastatic human breast tumor cells (MCF-7), we have found that overexpression of CD44v2,Δv3-10 in MCF-7 promotes these cells to undergo both rapid cell growth (Figure 6) and active cell migration (table 1). These findings suggest that this new breast cancer-specific CD44v2,Δv3-10 may play an important role in regulating breast tumor cell behaviors. Future work using a larger number of metastatic breast cancer samples is needed in order to further establish these newly identified CD44v3-containing isoforms as potential markers for monitoring the progression of human breast cancer metastasis.

6. ACKNOWLEDGMENT

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