

RESEARCH COMMUNICATION

NDRG2 Expression Regulates CD24 and Metastatic Potential of Breast Cancer Cells

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Abstract

Breast cancer is the most common malignancy in women in the world. High incidence and poor clinical outcomes underly the need for a better understanding of its tumor biology and how to effectively inhibit tumor progression. In the present study the question of whether NDRG2 might be a useful target for breast cancer therapy was addressed. With the increase or decrease of NDRG2 levels in MCF-7 and Bcap-37 cells by adenovirus-NDRG2 infection or NDRG2 siRNA transfection, CD24 expression was significantly decreased or increased, respectively. Furthermore, NDRG2 overexpression suppressed breast cancer cell adhesion and invasion, whereas knockdown of NDRG2 promoted these events. In conclusion, the data from the current study indicated that NDRG2, the product of a tumor suppressor gene, can regulate CD24 expression to decrease the metastatic potential of breast cancer cells.

Keywords: Breast cancer - NDRG2 - CD24 - metastatic potential

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Introduction

Breast cancer comprises more than 10% of all cancer incidences among women, accounting for the second most common type of cancer and the fifth most common cause of cancer death in the world. Despite advances in screening techniques to detect early stages of breast cancer and remarkable improvement in treatment outcomes, many women still develop into metastatic disease and ultimately die (Perou et al., 2000; Lerebours and Lidereau, 2002; Popescu and Zimonjic, 2002). Invasiveness and metastases of breast cancer cells are closely linked with poor prognosis and death. Molecules capable of inhibiting metastasis are attractive candidates for targeted therapies, and represent a critical aspect of future research.

One of the genes implicated in breast cancer development is NDRG2, a member of the N-myc downstream-regulated gene family that belongs to the alpha/beta hydrolase superfamily. NDRG2 was initially identified in our laboratory and was demonstrated to be involved in cell growth, differentiation, stress and hormonal responses (Shen et al., 2008; Wang et al., 2008). Recently, NDRG2 has been reported to act as a tumor suppressor gene (Li et al., 2002; Hu et al., 2004; Lusi et al., 2005; Liu et al., 2007; Zhao et al., 2008; Shon et

al., 2009). Its role in breast cancer has also been studied. It has been demonstrated that breast cancer has a low or undetectable levels of NDRG2 compared with the high expression of NDRG2 in normal and cancerous tissues. Further studies have found that NDRG2 is able to inhibit proliferation and enhance apoptosis of breast cancer as well as many other malignant tumors (Deng et al., 2003; Liu et al., 2007; 2008; Lorentzen et al., 2007; Wang et al., 2008). In addition, NDRG2 could induce BMP-4 expression and inhibit the metastatic potential of breast cancer cells, especially via suppression of MMP-9 activity (Shon et al., 2009). These data indicated an association exists between NDRG2 expression and reduced breast cancer malignant behaviors. However, to date, detailed mechanisms by which NDRG2 inhibited the aggressive behaviors of breast cancer are not fully explored.

When searching for the genes that are contributed to the NDRG2-mediated tumor inhibition, we estimated the relevance between NDRG2 and some adhesion molecules, which were important in breast cancer metastasis. We found that CD24 acted significantly as one such candidate. CD24 has been described as a diagnostic molecular marker of malignant tumor and for patient prognosis (Lim 2005). It is involved in cell adhesion and tumor metastasis. High rates of CD24 in breast cancer and many other cancers

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such as epithelial ovarian cancer, non-small cell lung cancer, prostate cancer, urothelial carcinoma, bladder cancer and gliomas are significantly associated with a more aggressive behavior (Senner et al., 1999; Kristiansen et al., 2004; Choi et al., 2007). In immunohistochemistry-based studies, cytoplasmic CD24 expression has a strong prognostic value. The staining intensity of breast tumors was correlated with lymph node metastasis (Lim and Oh, 2005). Furthermore, in breast cancer, CD24 mediates progression, metastasis and rolling of tumor cells through interactions with P-selectin (Aigner et al., 1998).

In this study, we have identified NDRG2 as a regulator that modulates the process of breast cancer cell adhesion and invasion. Through adenovirus infection-mediated overexpression of NDRG2 or siRNA-mediated NDRG2 downregulation in breast cancer cell lines, we verified that NDRG2 can decrease the malignant behaviors of breast cancer by altering the expression of CD24, a molecular diagnostic marker of malignant tumors.

Materials and Methods

Cell lines and culture

The human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (Manassas, VA, USA). Bcap-37 was from the Cancer Cell Repository (Second Affiliated Hospital, School of Medicine, Zhejiang University, China). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Hangzhou, China) at 37°C in the presence of 5% CO₂.

Gene infection

The multiplicity of infection (MOI) was determined for MCF-7 cells before the experiments. MCF-7 cells were seeded in 6-well plates at a density of 5×10⁵ cells/well and incubated overnight to reach approximately 80% confluency. Infection with adenoviruses carrying NDRG2 (Ad-NDRG2) or the negative control LacZ (Ad-LacZ) were then carried out for 2 h in DMEM medium, following medium replacement with fresh DMEM medium supplemented with 10% FBS for 48 h.

Gene transfection

Similarly, Bcap-37 cells were seeded in 6-well plates at a density of 5×10⁵ cells/well overnight followed by transfection with siRNA-NDRG2 or the negative control siRNA-NC using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The sequences of NDRG2 siRNA were: 5'-GCUCUCUGGAAUUCUGAGUUGAUA-3' (sense) and 5'-UUAAGAGCAUAUCUCGCCAGGAUGU-3' (antisense). The negative control siRNAs were: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). Transfected cells were cultured in DMEM for 6 h and then replaced with DMEM containing 10% FBS for 48 h.

RNA isolation and RT-PCR

Total RNA was isolated from the cells using

Trizol Reagent (Invitrogen) and quantified. Total RNA (5 µg) was then reverse transcribed into cDNA using an AMV reverse transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The cDNA was then subjected to PCR amplification using the following primers: NDRG2, 5'-ATGGCGGAGCTGCAGGAGGTGC-3' (sense) and 5'-TGAGGAACGAGGTCTGGGTGGG-3' (antisense); CD24, 5'-ACCTGTTTCCATTCAACAAGAGCAC-3' (sense) and 5'-TCTGAGATCGCACCCTGAC-3' (antisense); β-actin: 5'-GATCATTGCTCCTCCTGAGC-3' (sense) and 5'-TGTGGACTTGGGAGAGGACT-3' (antisense). The PCR mixture (25 µl) containing a Taq polymerase from Promega was first denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were separated in 3% agarose gels by electrophoresis and visualized with ethidium bromide under a UV light.

Quantitative RT-PCR

The cDNA (see above) was used as a template for real-time quantitative PCR analysis. Reactions were carried out in a Prism 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA) and using a Universal Mastermix (ABI). The primers were designed by use of the Primer Express Software (ABI). The primers were: NDRG2, 5'-GCCCAGCGATCCTTACCTACC-3' (sense) and 5'-GGTGCCCAATCCATCCAACC-3' (antisense); CD24, 5'-ACCTGTTTCCATTCAACAAGAGCAC-3' (sense) and 5'-TCTGAGATCGCACCCTGAC-3' (antisense); β-actin, 5'-GATCATTGCTCCTCCTGAGC-3' (sense) and 5'-TGTGGACTTGGGAGAGGACT-3' (antisense). The PCR reaction consisted of 12.5 µl of SYBR Green PCR Master Mix, 300 nM each of forward and reverse primers, and 1.5 µl template cDNA in a total volume of 25 µl. The thermal cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 34 s. To verify that the primer pair used produced only a single product, a dissociation protocol was carried out after thermocycling, thus determining dissociation of the PCR products from 65°C to 95°C. All samples were in triplicate and the data were normalized to the β-actin as a loading control.

Cell lysis and western blot analysis

The cells were washed twice with ice-cold PBS and then lysed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 0.5% NP-40, 0.1 mM PMSF and protease inhibitor cocktail. A total of 20 µg of cell lysate (as measured by the BCA protein assay; Pierce, Rockford, IL, USA) was loaded onto 12% SDS polyacrylamide gels for separation by electrophoresis and transfer onto Hybond nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). After that, the membranes were incubated with 5% fat-free milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at 37°C, and then with a primary antibody overnight at 4°C. The primary antibodies used were anti-NDRG2 (Abnova, Taiwan, China), anti-CD24 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin (Boster, Wuhan, China). After washing with

TBST three times, the membranes were incubated with a horseradish peroxidase-conjugated goat antimouse IgG antibody (Sigma) for 1 h and the blots were then developed with chemiluminescence substrate solution (Pierce) and exposed to X-ray film for visualization.

Adhesion assay

Twenty-four-well plates were coated with collagen I (5 µg/cm²). The cells after adenovirus infection or siRNA transfection were seeded at a density of 1x10⁵/ well and incubated for 80 min. Then the plates were washed twice with PBS to remove any non-adherent cells. The remaining cells were counted under microscopy for five randomly chosen fields per cm² of substrate surface area. Five duplicate wells were set up for each group and the experiments were repeated three times. The data were summarized as mean ± SD.

Invasion assay

In vitro invasion assays were performed using a 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA) with Matrigel pre-coating. The cells after adenovirus infection or siRNA transfection were seeded in the upper chamber of the Transwell at 1x10⁵ cells in 500 µl of serum-free medium, while the bottom chamber was fed with 200 µl medium containing 10% FCS. After the cells were incubated for 24h, the Transwells were fixed with methanol for 15 min and stained with gentian violet for 10 min. The cells in the upper chamber were removed by cotton swab and the cells that invaded through the Matrigel to the other side of the filter were manually counted. The experiments were performed in triplicate and the data represent the average of the total number of cells from three filters.

Statistical analyses

The data were summarized as mean ± standard deviation (SD). All statistical analyses were performed using one-way ANOVA. *P* < 0.05 was considered statistically significant.

Results

NDRG2 and CD24 expression in breast cancer cells

We first examined NDRG2 and CD24 expression in two different breast cancer cell lines, Bcap-37 and MCF-7, which were widely used as *in vitro* research for breast cancer. The results showed that expression of NDRG2 mRNA and protein is lower in MCF-7 cell line than that of Bcap-37 cell line, while CD24 expression is higher in MCF-7 cells than that in Bcap-37 cells (Figure 1).

Regulation of CD24 expression by NDRG2 of breast cancer cells

To further explore the relationship between NDRG2 and CD24, we first infected MCF-7, which expressed a low level of NDRG2, with adenoviruses carrying NDRG2 cDNA and found that NDRG2 mRNA and protein levels were up-regulated in these cells (Figure 2AC); moreover, levels of CD24 mRNA and protein were down-regulated in these cells (Figure 2BC). In contrast,

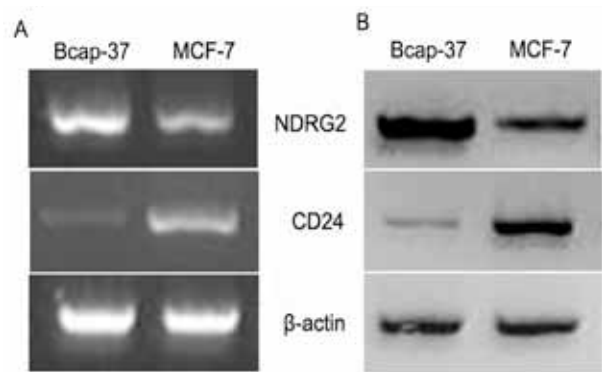


Figure 1. NDRG2 and CD24 Expression in MCF-7 and Bcap37 Cells. Adherent MCF-7 and Bcap-37 Cells were collected for extraction of RNA and protein and analysed for NDRG2 and CD24 expression using RT-PCR (A) and Western blotting (B). Data are representative of three independent experiments

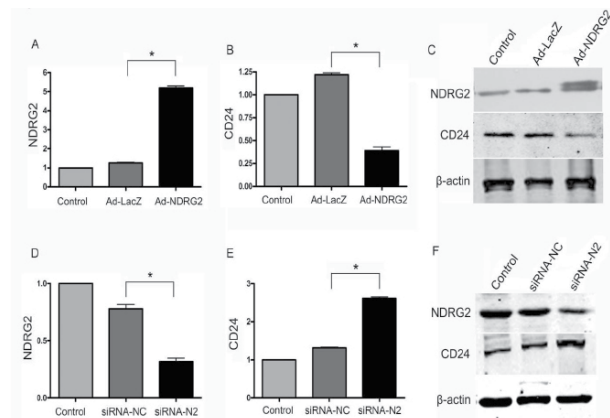


Figure 2. NDRG2 Modulation of CD24 Expression in Breast Cancer Cells. NDRG2-Low Breast Cancer Cells MCF-7 were Infected with the Adenovirus Carrying NDRG2 (Ad-NDRG2) or the Negative Control LacZ (Ad-LacZ), While NDRG2-High Breast Cancer Cells Bcap-37 were Transfected with NDRG2 siRNA or the Negative Control siRNA-NC Using Lipofectamine 2000. Afterwards, RNA and Protein from These Cells were Extracted and Subjected to qPCR (A, B, D and E) and Western Blot Analysis (C and F). β-actin was Used as a Loading Control. In A, B, D and E, the Histograms Represent the Average with Error Bars Showing the SD. Data Represent Three Independent Experiments. **P*< 0.05

after the NDRG2-highly expressed breast cancer cell line Bcap-37 was transiently transfected with NDRG2 siRNA oligonucleotides, NDRG2 expression was knocked down in this cell line (Figure 2DF); as a result, we found that CD24 expression was induced in these cells (Figure 2EF).

Modulation of breast cancer cell adhesion and invasion by NDRG2

Because CD24 acts as an adhesion molecule for P-selectin and is related to tumor metastasis, we therefore determined whether NDRG2 can modulate adhesion and invasion of breast cancer cells through altered CD24 expression. After over-expression and knockdown of NDRG2 expression in breast cancer cells, we observed that over-expression of NDRG2 significantly inhibited MCF-7 cells adhesion and invasion capacities (Figure 3A-D), while NDRG2 siRNA-infected Bcap-37 cells showed an

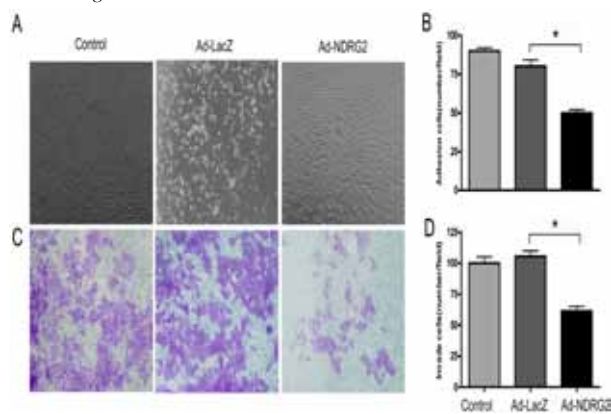


Figure 3. NDRG2 Suppression of MCF-7 Cell Adhesion and Invasion. NDRG2-Low Breast Cancer Cell Line MCF-7 was Infected with Adenovirus Carrying NDRG2 (Ad-NDRG2) or the Negative Control LacZ (Ad-LacZ). Afterwards, These Cells were Subjected to Adhesion (A) and Invasion Assays (C). In B, the Histogram Represents the Number of Adhesion Cells/Field with Error Bars Showing the SD; In D, the Histogram Represents the Number of Invade Cells/Field with Error Bars Showing the SD. Data Represent Three Independent Experiments. *P<0.05

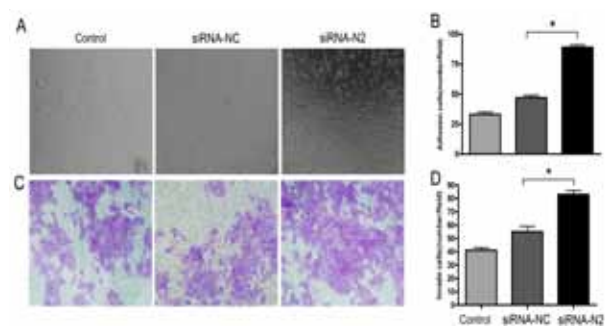


Figure 4. NDRG2 Suppression of Bcap-37 Cell Adhesion and Invasion. NDRG2-High Breast Cancer Cell Line Bcap-37 was Transfected with NDRG2 siRNA or the Negative Control siRNA-NC Using Lipofectamine 2000. Afterwards, These Cells were Subjected to Adhesion (A) and Invasion Assays (C). In B, the Histogram Represents the Number of Adhesion Cells/Field with Error Bars Showing the SD; In D, the Histogram Represents the Number of Invade Cells/Field with Error Bars Showing the SD. Data Represent Three Independent Experiments. *P<0.05

induction of tumor cell adhesion and invasion capacities, as compared to the control cells (Figure 4A-D).

Discussion

NDRG2 was expressed in many normal tissues, including mammary glands, but its expression was low or undetectable in clinically aggressive cancers such as breast cancer, glioma, gastric cancer, colon cancer, glioblastoma, thyroid, and renal clear cell carcinomas. Previous studies showed that NDRG2 could inhibit the growth and proliferation of these tumor cells (Li et al., 2002; Hu et al., 2004; Lusi et al., 2005; Liu et al., 2007; Zhao et al., 2008). In addition, the role that NDRG2 acts as a metastatic suppressor has also been reported. NDRG2 could significantly suppress aggressive hepatic carcinoma, fibrosarcoma and melanoma cells invasion activities via

antagonize transforming growth factor β 1 or suppress of NF-kappaB activity, respectively (Lee et al., 2008; Kim et al., 2009). As to breast cancer, it has been demonstrated that NDRG2 could induce BMP-4 expression and inhibit the metastatic potential of breast cancer cells mainly via suppression of MMP-9 activity (Shon et al., 2009).

Based on these results and our unpublished data, which has indicated that NDRG2 can regulate the expression of CD24 in hepatocellular carcinoma, we investigated if NDRG2 can modulate CD24 expression in breast cancer cells. We assessed the relationship between NDRG2 and CD24, as well as their effects on adhesion and invasion capacity of breast cancer cells and found that adenovirus carrying NDRG2 cDNA was able to inhibit CD24 expression and, in turn, suppress breast cancer cell adhesion and invasion capacity. As confirmation of these findings, NDRG2 siRNA yielded the opposite effects in breast cancer cells. Knockdown of NDRG2 expression using NDRG2 siRNA in Bcap-37 cells was found to induce CD24 expression and cancer cells adhesion and invasion capacity.

CD24 is a glycoprotein expressed at the surface of most B lymphocytes and differentiating neuroblasts. It acts as an adhesion molecule for P-selectin and is related to tumor growth and metastasis (Kim et al., 1997; 1998). The CD24/P-selectin binding pathway facilitates interactions with platelets or endothelial cells key to the dissemination of tumor cells, including breast cancer cells (Aigner et al., 1997; 1998). Moreover, enhanced CD24 expression is able to support rapid cell spreading and increase tumor cell proliferation, adhesion to fibronectin, collagens I and IV, and laminin through the activation of α 3 β 1 and α 4 β 1 integrin activity (Baumann et al., 2005). It has been demonstrated that CD24 is important for progression, migration, and metastasis of human breast cancer (Kristiansen et al., 2003). CD24 cross-linking induced apoptosis and inhibited migration in MCF-7 breast cancer cells (Kim et al., 2008). Thus, theoretically, molecules that can decrease CD24 expression will result in inhibition of breast cancer and could be novel targets for breast cancer treatment.

In the current study, we showed that CD24 suppression is sufficient to inhibit adhesion and invasion of breast cancer cell lines. At the same time we found that NDRG2 can regulate the CD24 expression. Based on previous facts and our study, we interfered that CD24 is a functionally relevant biomarker to predict aggressive behavior in breast patients, especially in those who also showed a low level of NDRG2. Furthermore, NDRG2 can act as a breast tumor suppressor by regulating different downstream molecules, which could then in turn lead to a more powerful effects on expression.

In conclusion, our data showed for the first time that NDRG2 was able to inhibit CD24 expression, which in turn decreased the adhesion and invasion capacities of breast cancer cells. In addition, our results suggest that in vivo studies are merited to determine whether NDRG2 inhibited metastasis of breast cancer through regulating CD24 expression. This mechanism may prove to be a novel approach towards more effective control of breast cancer in clinic.

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