NDRG2 correlated with favorable recurrence-free survival inhibits metastasis of mouse breast cancer cells via attenuation of active TGF-β production

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NDRG2 has been widely implicated in tumor cell growth of tumor cell (9), differentiation of dendritic cells (10), and apoptosis (11). NDRG2 attenuates cell proliferation through down-regulation of activator protein 1 (AP-1) activity in human colon carcinoma cells (4) and inhibited metastasis through suppression of nuclear factor kappa B activity (3). NDRG2 antagonizes transforming growth factor β (TGF-β)-mediated hepatocarcinoma cell invasion (12) as well as T-cell factor β-catenin signaling in human colon carcinoma (13).

Metastasis is the major cause of lethality among patients with cancer, including patients with breast cancer (14, 15). Unfortunately, targeted therapy against metastatic tumors has not been clinically available because the molecular mechanism underlying metastasis is not clear (16). Therefore, identifying genes that functionally inhibit metastasis and understanding their molecular mechanisms is a key in the cancer research.

In this study, we found that protein levels of NDRG2 were correlated with clinical prognosis of breast cancer. NDRG2 expression in breast cancer tissue was associated with favorable recurrence-free survival, and loss of expression resulted in increased lymph node metastasis and mortality in patients with breast cancer. To test the antimetastatic effects of NDRG2 in vitro and in vivo, we established an NDRG2-overexpressing mouse tumor cell line (4T1). This cell line (4T1-NDRG2) showed less ability to invade in vitro and metastasize in vivo. The transcript levels of PAI-1 and integrin β6 were down-regulated in 4T1-NDRG2 cells, and this down-regulation was associated with a reduction in the expression of bioactive autocrine TGF-β, which could induce the invasiveness of 4T1. To summarize, NDRG2 is a negative regulator of metastasis and a prognostic marker for recurrence of breast tumor.

Materials and methods

Patients and samples

The biospecimens for this study were provided by the Biobank of Chonbuk National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. A total of 189 patients were diagnosed and received their first line treatment at Chonbuk National University between January 1997 and January 2005. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols. All of the 189 breast carcinoma tissues and paired normal breast tissues using immunohistochemistry. Histological and clinicopathological data were correlated using Pearson’s chi-square test of independence. NDRG2 expression in human breast cancer tissues was inversely associated with lymph node metastasis and pTaN stage. Furthermore, patients with breast cancer with a high level of NDRG2 expression showed favorable recurrence-free survival (P = 0.038). To study the effect of NDRG2 on metastasis in vivo, we established an NDRG2-overexpressing mouse breast cancer cell line (4T1-NDRG2) and measured the metastasis and survival of 4T1-NDRG2 tumor-bearing mice. To test whether transforming growth factor β (TGF-β)–mediated metastasis of 4T1 was inhibited by NDRG2 expression, TGF–Smad-binding element (SBE)–luciferase activity and/or measurement of active TGF-β were performed in cell or tumor tissue level. 4T1-NDRG2 cells grew gradually and showed less metastatic activity in vitro. 4T1-NDRG2 cells showed lower SBE–luciferase activity and lower level of active autocrine TGF-β than 4T1-Mock did. Correctly, our data show that NDRG2 significantly suppress tumor metastasis by attenuating active autocrine TGF-β production, and the attenuation might be typically associated with the favorable recurrence-free survival of patients clinically.

Immunohistochemistry

Tissue specimens were cut into 4-µm-thick serial sections and mounted on charged glass slides (Superfrost Plus; Fisher Scientific). In brief, tissue sections were microwaved twice for 10 min in citrate buffer (pH 6.0) for antigen retrieval. Thereafter, the sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity followed by incubation with 1% BSA. The tissue sections were incubated with mouse monoclonal antibodies against NDRG2 (1:100; Abnova) overnight at 4°C in a wet chamber. Subsequently, the sections were stained using a standard EnVision-HRP kit (Dako) and developed with diaminobenzidine as a substrate. An irrelevant mouse immunoglobulin G of the same isotype as the primary antibody dilution solution served as a negative control.

Assessment of immunostaining

We evaluated the intensity of immunohistochemical staining relative to the staining intensity of adjacent ductal epithelium within the same section as
NDRG2 attenuates active TGF-β production in breast cancer

Cell invasion assay
4T1 cell invasion was tested using a modified Boyden chamber assay. Cells were allowed to grow to subconfluency (~70–80%) and were serum-starved for 24 h prior to detachment with trypsin/EDTA and washing with Dulbecco’s modified Eagle’s medium containing 1% fetal bovine serum, the cells (1.5 × 10^5 cells/300 μl) were added to the upper chamber of QCM™24-well cell invasion assay inserts (Millipore) kit and incubated for 48 h and then analyzed according to the manufacturer’s instructions.

Luciferase reporter gene assays
4T1 cells were transiently transfected with SBE–firefly luciferase vector and the pNull–Renilla luciferase vector in the absence or presence of TGF-β. Sometimes, HEK293 cells were transiently cotransfected with either pDNA3.1/hygro vector or pDNA3.1/hygro-NDRG2 vector, SBE–firefly luciferase vector, and pNull–Renilla luciferase vector. Afterwards, the cells were stimulated for 24 h with TGF-β1 (2 mg/ml) or culture supernatants obtained from 4T1-Mock or 4T1-NDRG2 cells. The cell lysates were assayed for firefly luciferase and Renilla luciferase activity as a measure of SBE activity by using the Dual-Glo® Luciferase Assay System (Promega). Each reporter assay was performed three times in triplicate. The results are presented as the mean ± SEM.

Measurement of active TGF-β
To measure amount of active TGF-β from cells or tissue, culture supernatants or tissue extracts were analyzed by a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (eBioscience). The supernatants were harvested from 4T1-Mock or 4T1-NDRG2 cultured in serum-free media for 3 days. In serum-free media, the proliferation rate of the cells did not show the difference. Assessed active TGF-β levels from tissues were normalized to total protein content as determined by Bradford assay (Bio-Rad Laboratories).

Statistical analysis
Statistical analysis was performed using the SPSS software package (version 14.0; SPSS Inc.). The correlation between histological index scores and other categorical clinicopathological factors was analyzed using Pearson’s chi-square test of independence. Recurrence-free survival was defined as the time from the date of surgery to the first date of recurrence of cancer or death from any cause. Overall survival was defined as the time from the date of surgery to the date of the last follow-up or death from any cause. The median follow-up period for all patients was 80.2 months (interquartile range, 61.9–92.6). Survival curve and median survival curve were estimated by the Kaplan–Meier method. Log-rank test was used to evaluate the statistical significance of differences in survival distribution. Multivariate analysis was performed using the Cox proportional hazard regression analysis. Cox proportional hazard model that included variables from the chi-square test for factors affecting survival and age variables were identified. All other data were analyzed using unpaired Student’s t-test. Results were considered statistically significant if P < 0.05.

Results
Correlation between NDRG2 expression levels and clinicopathological characteristics
The immunohistochemical staining result of NDRG2 in breast cancer tissues is shown in Figure 1A–C. Although NDRG2 has been known as a candidate of tumor-suppressor, some breast tumor tissues expressed NDRG2. To analyze whether there is any correlation between NDRG2 expression level and clinical meaning, the clinical and pathological characteristics of the 189 breast cancer patients who had undergone surgical resection are summarized in Table 1. The median age at the time of resection was 47 years; the median age of patients with a low level and a high level of NDRG2 expression was 46 and 48 years, respectively. High NDRG2 expression levels were observed in 69 (36.5%) of the 189 patients. When we tested for an association between the NDRG2 expression levels and the other categorical clinicopathological factors, we found that lymph node metastasis (P < 0.001), pTNM (tumor, node, and metastasis) stage (P = 0.011), and progesterone receptor status (P = 0.011) were significantly associated with NDRG2 expression status (Table 1). Importantly, patients with low NDRG2 expression levels showed significantly more frequent nodal metastasis and advanced tumor stage than those with a high NDRG2 expression levels.

Measurement of in vivo tumor growth and metastasis
Control (4T1-Mock) and NDRG2-expressing 4T1 (4T1-NDRG2; 5 × 10^5) cells were reseeded in sterile phosphate-buffered saline (200 μl) and subcutaneously injected into the dorsal flanks of BALB/c mice. Tumor dimensions were measured using a caliper and recorded every 4 days for 24 days. Tumor volume was calculated according to the following equation: (maximal length) × (perpendicular width) × (height)/2. Primary tumors were surgically removed from 4T1-Mock- and 4T1-NDRG2-bearing mice on day 21 after injection of the tumor cells. On day 19 after the surgery, all the mice were killed, and the lungs were dissected. Thereafter, lung-derived single cells were harvested after digesting the tissue with collagenase (Sigma). The cells were serially diluted and cultured in the presence of antibodies (hygromycin) for 10 days, and the number of metastatic cells was determined by counting the number of colonies formed at each dilution. Sometimes, the lung tissues were fixed with Bouin’s solution (Sigma), and the nodules on the lung tissue were counted.
Fig. 1. Immunohistochemical expression of NDRG2 and Kaplan–Meier survival analysis as a function of NDRG2 expression status in patients with breast carcinoma. (A) NDRG2 was constitutively expressed in normal breast tissue. (B) In breast cancer tissue, NDRG2 was highly expressed on the cytosolic membrane of tumor cells. (C) In some breast cancer tissues, NDRG2 was not expressed in cancer cells; however, the adjacent normal breast tissue showed well-preserved NDRG2 expression. Magnification: 400×. (D) Cumulative recurrence-free survival differences between patients with high and low levels of NDRG2 expression. (E) Cumulative overall survival differences between patients with high and low levels of NDRG2 expression. Statistical analysis was performed using the log-rank test of the difference.

High NDRG2 expression correlates with favorable recurrence-free survival, but not with overall survival

We first carried out univariate analyses to examine whether the expression status of NDRG2 correlates with recurrence-free survival and overall survival of patients with breast cancer. In all, 38 (20.1%) of 189 patients presented recurrence during the follow-up period. At the end of the follow-up, 166 (87.8%) patients were alive and 23 had died. High NDRG2 expression levels were associated with favorable recurrence-free survival ($P = 0.038$) (Figure 1D). The mean recurrence-free survival for patients with high NDRG2 expression levels was 101.4 months (95% confidence interval [CI], 95.1–107.7), whereas the time was reduced to 94.0 months (95% CI, 86.8–101.3) for those with low levels of NDRG2. However, NDRG2 expression levels were not correlated with overall survival (Figure 1E). The mean values of the overall survival of patients with high NDRG2 levels, low NDRG2 levels, and of all of the patients was 105.2, 151.2, and 154.1 months, respectively. We carried out multivariate Cox proportional hazard analyses to assess the predictive value of NDRG2 expression status for recurrence-free survival and overall survival by adjusting for other potentially prognostic factors, including age, histological grade, pTNM stage, and hormonal status. The results confirmed a worse recurrence-free survival outcome in patients with low NDRG2 expression levels, yet the correlation did not reach statistical significance ($P = 0.071$). The relative risk (RR) of recurrence was about twice as high in patients with low NDRG2 levels (RR, 2.077; 95% CI, 0.939–4.593) than in those with high NDRG2 levels. In a multivariate Cox regression analysis, the independent prognostic factors that significantly associated with recurrence-free survival were histological grade ($P = 0.03$) (RR, 3.739; 95% CI, 1.140–12.265), progesterone receptor status ($P = 0.03$) (RR, 0.444; 95% CI, 0.212–0.930), and pTNM stage ($P < 0.001$) (RR, 4.276; 95% CI, 2.230–8.202) (Supplementary Table 1). Levels of NDRG2 were not predictive of overall survival.

NDRG2 attenuates tumor growth in vivo and in vitro

To investigate NDRG2 function in breast cancer in vivo, we established a mouse breast cancer cell line overexpressing NDRG2 (4T1-NDRG2) (Figure 2A). The control (4T1-Mock) and 4T1-NDRG2 cells were seeded at $1.5 \times 10^5$ cells/well in 6-well plates, and cell numbers were counted with a hematocytometer at the indicated time points. After incubation for 2 days, the number of 4T1-NDRG2 cells was reduced by approximately 2.5-fold as compared with the control 4T1-Mock cells (Figure 2B). Moreover, the size of the solid tumors was smaller on back of BALB/c mice subcutaneously injected with 4T1-NDRG2 cells than on back of BALB/c mice subcutaneously injected with 4T1-Mock cells (Figure 2C). These data indicate that NDRG2 overexpression in breast cancer attenuates tumor growth in vitro and in vivo.

NDRG2 attenuates tumor invasion in vitro and inhibits tumor metastasis in vivo

To investigate whether NDRG2 affects the invasiveness and metastasis of breast cancer cells, we performed an in vitro invasion assay. 4T1-NDRG2 cells exhibited less invasiveness (40–60% inhibition).
Fig. 2. NDRG2 attenuates tumor growth in vivo and in vitro. (A) Transcriptional and protein levels of NDRG2 in 4T1-WT, 4T1-Mock, and 4T1-NDRG2 cells were measured by RT-PCR and Western blotting, respectively. (B) Growth curves of transfected cells in vitro. Cells were plated at a low density (1.5 × 10^4 per well), and cell numbers were counted daily for 3 days. (C) A 4T1-NDRG2 and 4T1-Mock cells (5 × 10^5 cells) were injected subcutaneously into the dorsal region of BALB/c mice, as described in Materials and methods. Tumor dimensions were measured with a caliper on day 24 after tumor cell implantation. Tumor volumes were calculated using the following formula: (maximal length) × (perpendicular width) × (height) / 2. **P < 0.01, by t-test.
4T1-Mock produced more active TGF-β than 4T1-Mock (Figure 4D). Furthermore, the supernatant from 4T1-Mock enhanced the invasiveness of 4T1-NDRG2 cells (Figure 4F) like recombinant active TGF-β (Figure 4F). Taken together, NDRG2 did not inhibit intracellular TGF-β-mediated Smad signaling directly but rather inhibited the production of active TGF-β.

Discussion

Breast cancer originates from breast tissue, the inner lining of milk ducts, or the lobules supplying the ducts with milk. Breast cancer is one of the most common malignant tumors among women, accounting for 14% of the cancer deaths in 2008 (19). Patients with metastatic breast cancer have traditionally been considered incurable with conventional treatment, although the survival of patients with metastatic breast cancer has been slowly improving, as shown in recent studies (20–23). Actually, tumor metastasis has been considered the most important reason for breast cancer morbidity and mortality (24). Although the expression of NDRG2 messenger RNA was statistically down-regulated in breast cancer (25) and NDRG2-regulated metastasis of breast cancer cell line through the down-regulation of CD24 expression (26,27) and up-regulation of BMP-4 (28), the correlation between clinical and functional evidences on the NDRG2 expression of breast cancer has been currently unknown. In the present study, we showed that high NDRG2 expression correlated with favorable recurrence-free survival. When the relationship between the NDRG2 expression level and the clinicopathological factors potentially predictive of prognosis was examined, we found that patients with low NDRG2 expression levels showed more frequent nodal metastasis and advanced tumor stage than did those with high NDRG2. To improve the outcome for patients with breast cancer, advances in the understanding of the pathophysiology of breast cancer and identification of proteins and molecular pathways that affect key metastasis and survival mechanisms are needed. Accumulating evidence suggests that NDRG2 is an important factor in tumor progression and metastasis, although its molecular mechanism is poorly understood (29–31).

The 4T1 tumor cell line was derived from a spontaneously arising BALB/c mammary tumor and has been extensively used in a well-characterized metastatic model (32,33). We established NDRG2-expressing 4T1 cells (4T1-NDRG2) to investigate the mechanisms of breast cancer metastasis inhibition by NDRG2. 4T1-NDRG2 cells exhibited a low proliferative phenotype, similar to NDRG2-expressing colon cancer cell lines, and retarded tumor growth in vivo. In human colon cancer cell lines, NDRG2 decreased c-Jun phosphorylation at Ser63, thereby inhibiting the function of AP-1 as a transcription factor. The down-regulation of cyclin D1, which is a target gene induced by AP-1, has also been reported and might be a cause of NDRG2-mediated retardation of proliferation. In addition, NDRG2 overexpression inhibited the metastasis of 4T1 cells from the established local site into the lungs. Such NDRG2 overexpression was correlated with the clinicopathological data from human patients with breast cancer. TGF-β signaling has been believed to play an important role in metastatic spread of breast cancer cells (34,35). Actually, many aggressive or advanced tumors are resistant to the growth inhibitory activity of TGF-β, which is an important negative regulator that prevents cell proliferation in early lesions. On the other hand, TGF-β can activate metastatic pathways (34,36). In human hepatocarcinoma cells, NDRG2 overexpression suppresses TGF-β signaling, inducing PAI-1, and integrin α3 and inhibits metastasis of SK-Hep-1 cells (12). In this study, we also found decreased PAI-1 expression levels in NDRG2-overexpressing 4T1 cells. We tested whether NDRG2 inhibited TGF-β-mediated Smad signaling by using an SBE–luciferase system and found low
SBE–luciferase activity in 4T1-NDRG2 cells. Unlike in human hepatocarcinoma cells, the lower SBE–luciferase activity might be not attributed to NDRG2-mediated inhibition of TGF-β signaling. Indeed, NDRG2 was not able to inhibit luciferase activity in TGF-β-treated HEK293 cells cotransfected with an NDRG2 expression construct and the SBE–luciferase vector. In addition, to investigate whether NDRG2 inhibits production of functional TGF-β, culture supernatants from 4T1-Mock or 4T1-NDRG2 cells were used to treat SBE luciferase-transfected HEK293 cells. 4T1-Mock, but not 4T1-NDRG2, culture supernatants induced luciferase activity in SBE luciferase-transfected HEK293 cells, and the amount of active TGF-β was higher in 4T1-Mock than in 4T1-NDRG2 in vitro and in vivo. Exogenous recombinant TGF-β as well as supernatant from 4T1-Mock cell enhanced the invasiveness of 4T1-NDRG2, confirming that NDRG2 did not inhibit TGF-β signaling and inhibited active TGF-β production. Taken together, we concluded that NDRG2 might inhibit the metastasis of breast cancer cells by reducing the production of active TGF-β. We also found that the transcript levels of integrin α6 were reduced in 4T1-NDRG2 cells. Integrin α6 controls the switch between latent and active TGF-β by forming heterodimers with integrin αv, which when bound to the latency-associated peptides 1 and 3, lead to the activation of the latent precursor form of TGF-β1 and TGF-β3 (37,38). In several tumor cell lines, integrin α6 has been reported to function as a positive regulator for tumor progression, proliferation, invasion, and epithelial-to-mesenchymal transition (39–41). Finally, we suggested that NDRG2 improved the recurrence-free survival of patients with breast cancer via inhibition of metastasis following attenuation of active TGF-β production.

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