**DCC-mediated Inhibition of Progression in Breast Cancer**

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**Keywords:** DCC, Migration and invasion, Breast cancer, Metastasis.

**Abstract.** DCC (deleted colorectal carcinoma) has been implied in several studies and play a major role in tumorigenesis. The present study evaluated the expression level of DCC in both breast cancer tissues and cell lines and the clinical significance of DCC in predicting the prognosis of breast cancer. The expression level of DCC mRNA was lower in both breast cancers tissues and cells with potential to metastasize. The Boyden chamber assay demonstrated that migration and invasion were decreased in MDA-MB-231 cells after upregulated DCC expression. The expression level of migration- and invasion-related genes, MMP-1 and MMP-9, decreased obviously, while that of MMP-2, MMP-3, and MMP-7 was not affected. The level of TIMP1 was upregulated, while that of TIMP2 was unaffected. The level of DCC expression was lowered in high metastasis potential breast cancers in both cell lines and pathological tissues. The upregulated expression of DCC inhibits the migration and invasion of breast cancer cells by modulating the expression of MMP-1, MMP-9, and TIMP1.

**Introduction**

The metastasis and recurrence are the leading causes of cancer-related deaths in breast cancer worldwide \(^1\). Metastasis has been described as the migration of tumor cells from primary tumor, followed by intravasation, survival, extravasation of the circulatory system, and progressive colonization of a distant site\(^1\). Various Genes, which have been proved to promote breast cancer metastasis proliferation\(^2\). Thus, by studying and interfering the pathway transduced by these factors, a method to intervene the tumor from progressing could be identified.

DCC (deleted colorectal carcinoma) is a major tumor suppressor and expressed widely in a series of tissues of normal adult. Fearon et al. firstly discovered and identified the DCC gene localized at 18q21.3 on the chromosome site, while the colorectal carcinoma was investigated in 1990 \(^3\). Reportedly, the loss of heterozygosity (LOH) at 18q, encompassing the DCC gene, has been linked to several human cancers \(^4\). Subsequently, LOH of 18q, typically associated with the decreased DCC expression, was linked to several other types of cancers, such as neuroblastoma, hematological malignancies, and gastric, prostate, endometrial, ovarian, esophageal, breast, testicular, and glial cancers \(^5, 6\).

Sherman et al. used immunochemistry to detect the expression of DCC in 75 cases of primary breast cancer tissues \(^7\). After a follow-up study of 10 years, DCC was ablated in 45/75 cases of breast cancer; also, a correlation between DCC expression and tumor local
recurrence and distant metastasis was established. Thus, DCC was considered a prognostic indicator in breast cancer. However, quantitative analysis of DCC expression and its role in breast cancer progression is not yet elucidated. Thus, in this study, we quantitated the expression of DCC in both breast cancer tissues and cell lines. The statistical analysis of the expression of DCC and other prognostic indicators of breast cancer suggested DCC as the putative cancer indicator for clinical prognosis of breast cancer. We also discussed the mechanism underlying DCC-mediated inhibition of migration, invasion, and metastasis of breast cancer.

Materials and Methods

Patients and Tissue Samples

A total of 58 cases of primary breast cancers were assimilated by the Department of Breast Surgery of China-Japan Union Hospital of Jilin University in the Jilin province of China from July 2013 to September 2014. None of these patients received preoperative chemo-, radiation-, or endocrine therapy. The mean age of these cases was 49.3 years. The present study was approved by the Ethics Committee of Jilin University, and all the patients provided informed consent. The samples were frozen (-80°C) immediately after surgery and stored in a biological resources repository, according to the national ethical guidelines. The tumors were diagnosed and classified according to the American Joint Committee on Cancer, breast cancer TNM staging system, and the breast cancer histology and subtype classifications of World Health Organization [8, 9].

Cell Culture and Transfection

pcDNA3.1-eGFP-DCC was obtained from Addgene (#13031; MA, USA). The MCF-10A cell line was routinely cultured with growth medium for non-tumorigenic human breast cells [low-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin with glutamine and Na-pyruvate], whereas the MCF-7, BT-549, and MDA-MB-231 cell lines were cultured using the growth medium for tumorigenic human breast cells (high-glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin). 24 h before transfection, the cells were plated at a density of 105 cells/well in a 24-well plate and allowed to grow up to 80% confluency. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transient transfection according to the manufacturer’s protocol.

Immunohistochemistry

The tissue samples were sliced and subjected to immunohistochemistry staining as described previously [10]. The antibodies against ER, PR, HER2, Ki67, p53, and DCC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted according to the manufacturer’s instructions. The ultrasensitive TM S-P kit and 3,3′-diaminobenzidine kit were purchased from Maixin Biotechnology (Fuzhou, China). Subsequently, the sections were counterstained with hematoxylin and coverslipped. These stained tissue sections were reviewed and scored independently by two pathologists. The proportion of tumor cells was scored as described previously [10].
Real-time PCR

Total mRNAs were extracted from frozen tissues/cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol, and 1 μg was reverse-transcribed using the M-MLV reverse transcriptase kit (Promega, Madison, WI, USA). Next, these cDNA samples were subjected to quantitative PCR amplification using StepOnePlus™ Real-Time PCR System (TaKaRa, Dalian, China) that was performed on a LightCycler 2.0 apparatus (Roche, Meylan, France) utilizing the Light Cycler FastStart DNA Master SYBR Green I Kit (Roche). The primers were as follows: DCC: forward CTGTCTGTGGACCGAGGTTT and reverse GGTTGGTCTCTTCACCTCACAGA; GAPDH: CGGTGCTGAGTATGTCGTGGAGT and CCAAGAAGAAACTCGGGAAGGC; MMP1: CACCTCTTTGACTCAGATATG and ACTTCCTCCCCTTATGATTCC; MMP2: ACTGGTTGTTGGGAACCTCAAGAG and CAAGGTCATGTACAGAAGG; MMP3: TCCTCTATCTACATGGCGCCAAG and CGGCACCTGGCTAAAGAC; MMP7: CCGATGGGTAACGCTTCTTAGG and AGGTGATTACACTCAGCAGTACG; MMP9: TGGGTGAGCGACGCTGAAAGA and CATGGTGTCCTACGTTGCTGATA; TIMP1: CACCAGAGAACCCACCATGGC and CACTCTGCAGTTTGCAGG; TIMP2: TCTGGAAACGACATGTTT and GGTGAGGCTCTTGATGTTG.

Western Blotting

The total protein of samples was extracted from cells. An equivalent amount of total protein from cells lysis was electrophoresed by 8% SDS-PAGE and transferred to PVDF membrane. The membrane was probed with goat anti-DCC antibody (Santa Cruz Biotechnology), followed by horse-radish peroxidase conjugated anti-goat IgG (1:8000, Sigma-Aldrich, St. Louis, MO, USA). The immunocomplexes were visualized by ECL Plus Western blotting system (Amersham, Buckinghamshire, UK).

Boyden Chamber

Boyden chamber migration assays (8 μm pore size, Corning, NY, USA) and modified Boyden chamber Matrigel invasion assays (8 μm pore size, Corning) were performed according to the manufacturer’s instructions. A total of 3×10^4 cells were seeded into the top chambers for the migration assay. The top chambers were placed in the bottom wells that were filled with culture media and supplemented with 5% FBS. After 16 h, the non-migrating cells present on the upper surface of the insertion were swabbed with a sterile cotton swab. The cells migrated onto the lower surface of the filter were fixed and stained using 0.1% crystal violet. For invasion assay, the protocol was similar to that of migration assay except that the top chambers were coated with Matrigel (BD Bioscience, NJ, USA) before addition of the cells.

Results

DCC was Lowly Expressed in Both Breast Cancer Cell Lines and Tissues

Real-time PCR and Western blot results showed that DCC mRNA and protein were lowly expressed in breast cancer cell lines including MCF-7, BT-549, and MDA-MB-231 as compared to MCF-10A, which originates from normal breast epithelial cells. In addition, the expression of DCC mRNA in MDA-MB-231 cell lines was much lower than the other two breast cancer cell lines. (Fig. 1A, B).
Figure 1. A. Real-time PCR showed that the expression of DCC mRNA was significantly higher in MCF-10A cell line than any of the other three cell lines (n=5, P<0.05). In comparison to the MCF-7 and BT-549 cell lines, the mRNA expression of DCC was remarkably decreased in the MDA-MB-231 cell line (n=5, P<0.05). B. Western blot showed the same changes in DCC protein expression in different cell lines.

Utilizing 58 cases of clinical samples, we first detected the expression of DCC protein by immunohistochemistry staining of both breast cancer and its matched distant normal tissue samples. The results were scored as none, weak, moderate, and strong. The DCC protein was moderately expressed in the cytoplasm of the distant non-cancerous cells (Fig. 2A). Conversely, the DCC protein was weakly stained in the cytoplasm of breast cancer cells (49/58, 84.5%; Fig. 2A). Next, the real-time PCR in these tissue samples detected that the level of DCC mRNA was significantly lower in breast cancer tissues than that in the matched distant non-cancerous tissues. (53/58, 91.4%; Fig. 2B).

Figure 2. A. Immunohistochemistry staining of DCC protein showed a distinct decrease in the matched normal breast tissue samples as compared to the breast cancer tissues. B. 57/58 breast cancer patients showed a lower DCC protein expression in the cancer tissues than that in the matched normal breast tissues.
Association of DCC Expression with Clinicopathological Parameters from Breast Cancer Patients

Next, we associated the expression of DCC mRNA with the clinicopathological parameters from breast cancer patients. The results showed that DCC was lowly expressed in cases with metastasized lymph nodes as compared to those without lymph node metastasis (\(P<0.001\)) (Fig. 3). However, no association was observed between DCC expression and the other clinicopathological parameters, such as tumor size, number lymph node, vascular invasion status, breast cancer subtype, TNM stage, and biomarkers (ER, PR, HER-2, and Ki67) (Data not shown).

![Figure 3](image)

**Figure 3.** Real-time PCR showed that the relative expression of DCC mRNA in breast cancer tissue with lymph node metastasis was significantly lower than that in the breast cancer tissue without lymph node metastasis (\(P<0.001\)).

Mechanism of the Role of DCC in Inhibiting Migration and Invasiveness in Breast Cancers

In order to observe the role of DCC in breast cancer cells, we upregulated the expression of DCC with transfection of pcDNA3.1-eGFP-DCC in MDA-MB-231 cells. Next, we used the Boyden chamber assay to study the migration and invasive changes by upregulating the expression of DCC. The results showed that after upregulation of DCC, the migratory cell number declined by 41.2% as compared to the control group (\(n=5\), \(P<0.001\)) and 50.8% as compared to the pcDNA3.1-eGFP group (\(n=5\), \(P<0.001\)). The invasive cell number was reduced by 38.7% as compared to the control group, and this reduction was 45.7% as compared to the pcDNA3.1-eGFP group (\(n=5\), \(P<0.001\)) (Fig. 4).
Figure 4. The Boyden chamber assay showed that after the upregulation of DCC in MDA-MB-231 cells, the number of migrated and invaded cells was significantly reduced as compared to the other two control groups (n=5, P<0.01).

Finally, we detected the mRNA level of a series of matrix metalloproteinase (MMP) family members related to cell migration and invasiveness by real-time PCR. Herein, we found that the mRNA expressions of migration- and invasion-related genes, MMP-1 and MMP-9, decreased obviously, while that of MMP-2, MMP-3, and MMP-7 was not affected obviously (n=5, P<0.05). The mRNA level of TIMP1 increased after the upregulation of DCC (n=5, P<0.05), while that of TIMP2 was not affected remarkably (Fig. 5).

Figure 5. The mRNA expression of MMP-1 and MMP-9 after DCC upregulation was significantly decreased, while that of TIMP1 was remarkably increased (P<0.01).
Discussion

The prognosis of breast cancer was affected by a number of factors, which can be broadly classified into those related to the tumor stage, clinical variables, pathological features, and ontogenetic, molecular, and immunological variables. However, the stage, grade, and the status of estrogen receptor (ER) and progesterone receptor (PR) were the vital prognostic factors. LOH of chromosome 18q, which harbors the DCC gene, was identified in a large proportion of colorectal cancers \[11\]. DCC may be inactivated in different tumors including breast carcinoma; loss of 18q21 has been associated with a decreased survival in breast carcinoma patients \[12\]. However, little was known about the mechanism underlying DCC in breast cancer \[13\].

Originally, DCC was discovered as a putative tumor-suppressor gene in colorectal cancer, localized on chromosome 18q \[1\]. A tumor-suppressor role for DCC has been addressed in studies that failed to show a distinct malignant phenotype in DCC knockout mice models \[14\]. However, recent studies have challenged this theory and favored a role for DCC in suppressing the tumor growth and metastasis \[1\]. The present study aimed to investigate the expression status of DCC mRNA quantitatively and discuss its role in breast cancer during tumor progression. Firstly, we found that the mRNA level of DCC was less expressed in breast cancer tissues as compared to the adjacent non-cancerous tissues, which was in agreement with that of Koren et al \[7\]. Furthermore, we compared the expression of DCC with common parameters of breast cancers including pathological stage, grade, ER and PR expression, and other biomarkers of breast cancer. This study revealed that the lymph node metastasis-positive tumors were associated with a low expression of DCC mRNA. However, no significant correlation was established between DCC status and other prognostic parameters such as pathological stage, grade, and ER and PR expression. Koren et al. pointed out significant differences between cases without metastasis or local recurrences as compared to those with metastasis or local recurrences in breast cancer \[7\]. Shin et al. also found that DCC loss occurred in later stages of multistep colorectal carcinogenesis \[15\]. These studies implied that DCC could be an indicator of metastasis.

In order to illustrate the mechanism of DCC in breast cancer, we upregulated the expression of DCC in MDA-MB-231 cells and observed that the decreased migration and invasion ability might be caused by the low expression of MMP-1 and MMP-9 and high expression of TIMP1. MMPs belong to a multigene family of endopeptidases that are physiologically relevant mediators of extracellular matrix (ECM) degradation. These are critical enzymes that participate in several physiological and pathological conditions by degrading the ECM molecules (for example, collagen, laminin, and fibronectin) and releasing the cryptic epitopes from the ECM \[16\]. Reportedly, MMPs regulated the neuronal migration and neurite outgrowth by interacting with DCC in the neural system \[17\]. Moreover, the activity of MMPs is tightly regulated by interaction with tissue inhibitor of metalloproteinases (TIMPs). TIMPs are 20–30 kDa secreted proteins that form tight but relatively low selectivity 1:1 complexes with the active forms of metzincin proteases \[18\].

Lee et al. found that Netrin-1 requires DCC/PKCα for activating the ERK/JNK/NF-κB pathway to control the MMP-mediated E-cad downregulation in promoting the cell motility of mesenchymal stem cells \[19\]. The DCC-induced migration was also observed by Junge et al.\[20\], who demonstrated that Netrin1/DCC was potentially involved in the migration of additional spinal cord neurons. Several pieces of evidence \[21, 22\] suggested that Netrin-DCC signaling can regulate and be regulated by the cAMP-dependent protein kinase, PKA. Taken together,
we inferred that DCC is a multifunctional receptor that could influence the tumorigenesis and progression in different tumors via several pathways. Therefore, this was inferred as one of the mechanisms underlying DCC in breast cancers.

**Conclusion**

The level of DCC expression declined in high metastasis potential breast cancers in both cell lines and pathological tissues. Thus, the upregulation of expression of DCC inhibited the migration and invasion of breast cancer cells by modulating the expression of MMP-1, MMP-9, and TIMP1.

**References**


