Abstract. Deleted in breast cancer 1 (DBC1) has been known to be overexpressed and serves as a poor prognostic indicator of several human cancers. In this study, we examined DBC1 expression in a total of 142 gastric cancer tissues by immunohistochemistry and revealed that DBC1 was overexpressed in gastric cancer and significantly associated with TNM stage and lymph node metastasis. The in vitro experiments showed that DBC1 expression correlated with the ability of anoikis resistance in gastric cancer cells, which has been defined as critical to metastasis. Furthermore, the results showed that the IKK-β/NF-κB signaling pathway was involved in the regulation of anoikis resistance by DBC1 in gastric cancer cells. Taken together, the results indicated that DBC1 promotes anoikis resistance in gastric cancer cells by regulating NF-κB activity and may thus be a new therapeutic target for preventing potential metastasis.

Introduction

Although much progress has been made in early diagnosis, surgery, chemotherapy and targeted drugs, gastric cancer remains the second most common cause of cancer-associated mortality worldwide (1,2). Metastasis is a major clinical obstacle for the treatment of gastric cancer and therefore, a better understanding of the gene regulation involved in the development of metastasis may lead to therapeutic improvements for gastric cancer patients.

Metastasis is a multistep process including the dissociation of cancer cells from primary sites, survival in the vascular system, and proliferation in distant target organs (3). As a barrier to metastasis, cells normally undergo an apoptotic process known as ‘anoikis’, a form of cell death due to loss of contact with the extracellular matrix or neighboring cells (3-5). However, a subset of cancer cells acquires the ability to resist anoikis and thus survives after detachment from the primary sites and travels through the circulatory and lymphatic systems to disseminate throughout the body (3-5). Recently, identification of the factors and mechanisms that control anoikis has become a high priority in cancer cell biology and developmental therapeutics.

Deleted in breast cancer 1 (DBC1) is a nuclear protein encoded by a gene on 8p21 that was originally believed to reside within a deleted region in breast cancer, a deletion assignment that was subsequently found to be inaccurate (6,7). DBC1 overexpression has been observed in colorectal, esophageal and breast cancer, where its overexpression correlates, in some cases, with poor prognosis (8-11). Currently, the molecular and cell functions of DBC1 are being extensively investigated to reveal its precise physiological role. The endogenous DBC1 is a nuclear protein and the amino-terminus of DBC1 has been shown to be a protein-interaction surface. Additionally, DBC1 serves as a transcriptional factor that represses the transcriptional activation function, such as SIRT1, BRCA1 and estrogen receptor β (12-15). However, DBC1 was recently reported to interact with IKK-β, stimulate its kinase activity, and thus promote NF-κB transcriptional activity through the phosphorylation of relA serine-536, by which, DBC1 suppressed anoikis in normal epithelial and breast cancer cell lines (7).

In this study, we performed immunohistochemical staining to examine the prevalence and prognostic impact of DBC1 expression in gastric cancer patients. Moreover, we investigated the possible role and mechanism of DBC1 in the regulation of anoikis in gastric cancer cells and demonstrated that DBC1 promoted anoikis resistance of gastric cancer cells by regulating NF-κB activity.

Materials and methods

*Contributed equally

Key words: deleted in breast cancer 1, anoikis, metastasis, NF-κB, gastric cancer

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Patients and samples. A total of 142 cases of gastric adenocarcinoma patients who had radical gastrectomy at the 82nd hospital of the PLA between January 2008 and December 2009 were included in the present study. None of the patients...
received chemotherapy prior to surgery and were followed-up by a review of medical records and telephone calls. Data on gender, age, histological type, TNM stage, metastatic status and the Lauren classification were collected from the medical record library of the hospital. This study was approved by the Ethics Committee of the 82nd hospital of the PLA, and informed consent was obtained from the patients in accordance with the Declaration of Helsinki.

**Immunohistochemical staining.** Immunohistochemical staining was carried out using a rabbit SP immunostaining kit (Zhongshan Goldenbridge Biotechnology, Beijing, China). Sections were dewaxed in xylene and dehydrated through a gradient concentration of alcohol. Then, sections were treated in a microwave for 10 min for antigen retrieval. After blocking the endogenous peroxidase and non-specific staining with 0.3% (v/v) hydrogen peroxide and normal goat serum, the sections were incubated with anti-DBC1 antibody (1:100 dilution, Bethyl Lab, Montgomery, TX, USA) overnight at 4°C. After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated biotinylated IgG for 30 min, followed by washing with phosphate-buffered saline (PBS). The sections were visualized by diaminobenzidine (DAB) solution and counterstained with hematoxylin (both from Zhongshan Goldenbridge Biotechnology). The sections incubated with rabbit IgG instead of primary antibody were used as negative controls.

**Staining evaluation.** Immunohistochemical staining was examined by two independent pathologists who were blinded to the clinicopathological information. Each case was evaluated to estimate the intensity of cell staining and the percentage of positive tumor cells. The intensity of cell staining was graded according to the following scale: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The extent of staining was evaluated by the percentage of positive tumor cells: 0 (negative), 1-4 (low expression), 5-8 (moderate expression, ++), and 9-12 (high expression, +). For each sample, the score for intensity was multiplied by the score for extent of staining to provide a final score. Therefore, the expression of DBC1 was defined as: 0 (negative, -), 1-4 (low expression, +), 5-8 (moderate expression, ++), and 9-12 (high expression, +++). For multiple lymph node metastases in a single case, an average immunohistochemical score was calculated to define the expression of DBC1 in metastatic lymph nodes.

**Cell culture and transfection.** Human MKN45 and MGC803 gastric cancer cell lines were cultured in modified Eagle’s medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were maintained at 37°C in a humidified 5% CO₂ incubator.

For transient transfection, the gastric cancer cells were transfected with lentivirus vector encoding the green fluorescent protein were used as controls.

**Anoikis analysis.** After a 48-h transfection, the gastric cancer cells were collected and continuously cultured in plates precoated with Poly-HEMA for 48 h (16,17). Subsequently, the cells were stained with Annexin V/PI and analyzed by flow cytometry.

**Luciferase analysis.** The gastric cancer cells were seeded in 6-well plates at a concentration of 1.0x10⁶/well. After 24 h, the cells were infected with lentivirus and treated with Bay 11-7082 (10 µM) or PDTC (50 µM) 24 h later. After another 24 h, cells were co-transfected with 1 µg of NF-κB luciferase reporter pNF-κB-luc WT and 50 ng of Renilla luciferase plasmid phRL-TK (Promega, Madison, WI, USA) using Lipofectamine 2000 transfection reagent (Invitrogen, USA). Reporter activities were analyzed using the Promega dual luciferase assay kit (Promega) according to the manufacturer’s instructions. The luciferase activity was normalized to the Renilla luciferase activity.

**Western blot analysis.** Total cell lysate was prepared in 1X SDS buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were then blotted with antibodies to DBC1 (Bethyl Lab), c-FLIP, bcl-xl and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antigen-antibody complexes were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

**RT-qPCR analysis.** The TaqMan stem-loop RT-PCR method was used to assess the expression of c-FLIP, bcl-xl and GAPDH with kits from Applied Biosystems (Foster City, USA). For relative expression levels, the 2(-∆ΔCt) method was used as previously described (18). Experiments were carried out in triplicate for each data point, and a data analysis was performed using Bio-Rad IQ software (Hercules, CA, USA).

**Statistical analysis.** Data are presented as means ± SEM. The Chi-square test was used to compare the differences in the DBC1 expression level with various clinicopathological parameters in gastric cancer. Survival curves were plotted by the Kaplan-Meier method and the log-rank test was carried out to compare differences in survival. Statistical analyses were performed using SPSS 17.0 software (Chicago, IL, USA). P<0.05 was considered statistically significant.

**Results**

*Increased expression of DBC1 is associated with lymph node metastasis in gastric adenocarcinoma.* Immunohistochemistry was performed to detect the expression of DBC1 in 142 cases of gastric adenocarcinoma tissues and the corresponding adjacent non-tumor tissues. DBC1 was detected primarily in the nuclei of cancer cells (Fig. 1). High expression (+++), moderate expression (++), low expression (+), and negative expression (-) of DBC1 were identified in 13, 32, 47 and 50 samples of the adenocarcinoma tissues, respectively, while in the adjacent non-tumor tissues, only a few cells with weak staining for DBC1 were observed in two cases. We also assessed the potential relationship between DBC1 expression and clinical characteristics of gastric adenocarcinomas. As shown in Table I, the expression of DBC1 was significantly correlated with TNM stage (P<0.05) and lymph node metastasis (P<0.05). However, there was no significant correlation between DBC1 expression...
and age, gender, distant metastasis, histological grade and the Lauren classification (P>0.05).

Given that DBC1 expression was significantly correlated with lymph node metastasis, we also detected the expression of DBC1 in lymph node metastasis and found that the expression level of DBC1 in the metastatic lymph nodes was concomitant with that in the primary gastric adenocarcinoma tissues (Linear correlation analysis for immunohistochemical scores, P<0.05). Moreover, in cases identified as low DBC1 expression, we found that the percentage of DBC1-positively expressed cancer cells in metastatic lymph nodes was markedly higher than that for the primary site (P<0.05, Fig. 1C).

The relationship between DBC1 expression and survival was also analyzed. The mean survival times in patients with high expression (+++), moderate expression (++), low expression (+) and negative expression (-) of DBC1 were 27.5, 38.0, 40.7 and 44.6 months, respectively. The Kaplan-Meier analysis (Fig. 2) revealed that DBC1 expression was significantly correlated with a shorter overall survival time (log-rank, Chi-square=8.551, P<0.05).

### Table I. Correlation between DBC1 expression and clinical characteristics of gastric cancer.

<table>
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<tr>
<th>Characteristics</th>
<th>No. of patients</th>
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<th>+</th>
<th>++</th>
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*P<0.05.

### DBC1 expression correlates with the ability of gastric cancer cells to resist anoikis.

Resistance to anoikis has been considered a hallmark of metastatic cancer cells, as it is required for anchorage-independent growth during tumor dissemination. Previous studies have identified that DBC1 is an important co-factor for the control of the IKK-β/NF-κB signaling pathway that regulates anoikis (7). Therefore, we hypothesized that DBC1 is involved in the metastasis of gastric cancer cells by mediating resistance to anoikis via IKK-β/NF-κB signal. We initially examined the effect of DBC1 expression on anoikis in gastric cancer cells. To achieve this, lentivirus-mediated DBC1 or DBC1 shRNA were transfected into MKN45 and MGC803 cells. As shown by western blot analysis in Fig. 3A, DBC1 protein levels in the MKN45 and MGC803 cells following transfection with lentivirus-mediated DBC1 or DBC1 shRNA were markedly higher than that for the primary site (P<0.05, Fig. 1C).

The specific down-regulation of DBC1 expression by lentivirus-mediated DBC1 shRNA induced an increased sensitivity to anoikis in MKN45 and MGC803 cells (P<0.05, Fig. 3A and B).

**IKK-β/NF-κB signaling pathway is involved in the regulation of anoikis by DBC1 in gastric cancer cells.** In this study,
Bay 11-7082, a highly specific inhibitor of IKK-β activity, and PDTC, a specific inhibitor of NF-κB activity, were used to assess the role of the IKK-β/NF-κB signaling pathway in the regulation of anoikis by DBC1 (19,20). Fig. 4A shows that in DBC1 upregulated gastric cancer cells (MKN45 and MGC803), the levels of NF-κB activation following detachment were significantly higher than that of the control cells (P<0.05). However, we also observed that in gastric cancer cells cultured in normal cell plates, DBC1 upregulation did not obviously influence the activation of NF-κB (P>0.05). Fig. 4B shows that NF-κB activation was significantly inhibited in gastric cancer cells following detachment after exposure of Bay 11-7082 or PDTC, and coincidently, the anoikis rates of gastric cancer cells were significantly increased. Fig. 4C shows that either Bay 11-7082 or PDTC reversed the increased anoikis resistance in DBC1-upregulated gastric cancer cells, which indicated that DBC1 contributed to the anoikis resistance by activating the IKK-β/NF-κB signaling pathway.

**DBC1 promotes anoikis resistance by regulating NF-κB-mediated transcription.** Among NF-κB target genes, c-FLIP and bcl-xl are particularly noted for their ability to regulate anoikis (7,21,22). We examined the mRNA and protein expression changes of c-FLIP and bcl-xl in DBC1 upregulated gastric cancer cells (MKN45 and MGC803). As shown in Fig. 5A and B, the mRNA and protein expression levels of c-FLIP and bcl-xl were significantly increased in DBC1 upregulated gastric cancer cells following detachment. We also examined the effects of Bay 11-7082 and PDTC on the expression of c-FLIP and bcl-xl. As expected, after exposure of Bay 11-7082 or PDTC, the improved mRNA and protein expression of c-FLIP and bcl-xl were reversed in the DBC1 upregulated gastric cancer cells, further indicating that DBC1 promotes anoikis resistance by regulating NF-κB-mediated transcription.

**Discussion**

DBC1 was first identified in 2002 by Hamaguchi et al (23). As a new transcriptional co-activator, DBC1 exhibits its function by modulating the activities of various proteins. Currently, the role of DBC1 in cell survival remains controversial. On the one hand, DBC1 has been revealed to be able to directly bind to the catalytic domain of SIRT1 and decrease the deacetylase activity of SIRT1, thus inhibiting SIRT1-dependent cell survival (12,13). On the other hand, DBC1 mediates endocrine-resistant breast cancer cell survival. It promotes
Figure 3. Effects of DBC1 expression on the sensitivity to anoikis in gastric cancer cells. (A) MKN45 and (B) MGC803 cells. Data are representative of five independent experiments.

Figure 4. DBC1 regulates anoikis via the IKK-β/NF-κB signaling pathway. (A) Left panel: DBC1 increased the level of NF-κB activation following detachment in gastric cancer cells; right panel: DBC1 did not increase the level of NF-κB activation in gastric cancer cells cultured in normal condition. (B) Inhibition of NF-κB activation by Bay 11-7082 or PDTC led to increased sensitivity of gastric cancer cells to anoikis. (C) Inhibition of NF-κB activation by either Bay 11-7082 or PDTC reversed the influence of DBC1 upregulation in the sensitivity of gastric cancer cells to anoikis. Data are representative of five independent experiments. *P<0.05 vs. other groups.
estrogen-independent proliferation and inhibits estrogen-independent apoptosis in estrogen receptor α positive breast cancer cells (24,25). DBC1 also represses the transcription of BRCA1 and inhibits its tumor-suppressor activity (14). Several studies also reported that DBC1 modulates cell growth by regulating the transcriptional activity of retinoic acid receptor α and androgen receptor in breast and prostate cancer cells (26-28). The abovementioned findings indicated that DBC1 may act as either a tumor promoter or a tumor suppressor in tumorigenesis, depending on the cell context and type of tumor.

Although DBC1 was initially termed as deleted in breast cancer 1, it was subsequently found to be overexpressed in breast cancer tissues in comparison with corresponding matched normal tissues, and that overexpression was correlated with clinicopathological factors, especially metastasis, and poor prognosis (10,11). DBC1 was then found to be overexpressed in various human cancers including esophageal cancer, colorectal cancer, soft tissue sarcomas and lymphoma (8,9,29,30). With regards to gastric cancer, Kang et al and Cha et al showed that DBC1 was overexpressed in gastric cancer tissues and could be used as a prognostic indicator for gastric cancer patients (31,32). In the present study, we found that DBC1 was significantly upregulated in gastric adenocarcinoma patients and this expression was significantly correlated with TNM stage and lymph node metastasis. Our results also support that DBC1 is a prognostic factor that is associated with poor prognosis in gastric cancer. In addition, we examined the expression of DBC1 in metastatic lymph nodes and found a higher expression that in the primary gastric cancer tissues, further indicating that DBC1 may play an important role in gastric cancer metastasis.

Anoikis, a special form of apoptosis occurring when cells detach from the extracellular matrix, is a critical mechanism in maintaining tissue homeostasis and development. Anoikis-resistance has been considered as a hallmark of metastatic cancer cells, especially because the anchorage-independent growth of cancer cells is a classic characteristic of different types of human malignancies (33). Among the signaling and transcription factor pathways involved in regulating anoikis, inflammatory-response transcription factor NF-κB is notable because it links anoikis with inflammatory signaling between and within cells (34,35). Recently, Park et al reported that DBC1 suppresses anoikis in both normal epithelial and cancer cells (breast cancer MCF10a cells) by regulating the IKK-β/NF-κB signaling pathway. DBC1 may be conceptualized as a co-factor for IKK-β that stimulates its kinase activity on relA (S536), promoting the transcriptional activation of NF-κB target genes such as c-FLIP and bcl-xl, which enhance anoikis resistance (7). Using gene transfection assays, we showed that DBC1 expression correlates with the ability of anoikis resistance in gastric cancer cells. Furthermore, by using Bay 11-7082 and PDTC, we demonstrated that the IKK-β/NF-κB signaling pathway is involved in the regulation of anoikis resistance by DBC1 in gastric cancer cells. Combined with the immunohistochemical results, we suggest that there is a subset of gastric cancer cells with a high DBC1 expression that can survive the detachment process for enhanced NF-κB activation, subsequently leading to metastasis.

In summary, our study has demonstrated that DBC1 is overexpressed in gastric cancer and associated with poor prognosis. Our study also provides experimental evidence that DBC1 promotes anoikis resistance in gastric cancer cells.
by regulating NF-κB activity, raising the possibility of using DBC1 as a new therapeutic target for preventing metastasis.

References