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RESEARCH

Dystroglycan is Associated with Tumor Progression and Patient Survival in Gastric Cancer

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Abstract Previous reports had indicated that there was a possible correlation of dystroglycan (DG) with biological behavior of cancer cells and cancer patients' survival. However, the role of DG expression in gastric cancer was rarely studied. In this study, α-DG and β-DG expression were determined by immunohistochemistry in specimens of primary cancer, metastatic lymph node, distal metastatic lesion, and their normal counterpart tissues in 20 gastric cancer patients. Correlations between α-DG and β-DG expression and prognosis were retrospectively analyzed. Our results found that positive expression of α-DG in normal mucosa, paired primary tumor, metastatic lymph node and distal metastatic site was detected in 95%, 70%, 25%, and 5% specimens, individually. Regarding β-DG,it was 70%, 55%, 10%, and 10%, individually. Patients who had lower α-DG expression in tumors than in normal counterparts showed poor survival (p=0.002), whereas such a correlation was not found in the case of β-DG (p= 0.079). Difference of α-DG between primary tumor and its normal counterparts was an independent prognostic factor in gastric cancer with distal metastasis. This study showed DG expression was gradually reduced during tumor progression. Different expression of α-DG, but not β-DG, between primary tumor and normal specimen, correlated with patient survival, implicating a potential marker for gastric cancer prognosis.

Keywords Adhesion molecule · α-dystroglycan · β-dystroglycan · Gastric cancer · Prognosis

Abbreviation

DG	dystroglycan
ECM	extracellular matrix
mLN	metastatic lymph node

Introduction

Gastric cancer is the most frequently diagnosed carcinoma in China and has the second highest cancer related mortality rate in the world [1]. Like many epithelial tumors, cell adhesion molecules mediates the interaction of epithelial cells with basement membrane, which regulates cell growth, motility, and differentiation by integrating signals from extracellular matrix (ECM) in gastric cancer [2]. Cell adhesion molecules alterations are critical for understanding mechanisms of tumorigenesis, enabling earlier diagnosis and development of more effective treatment.

Like other adhesion molecules, dystroglycan (DG) is a part of a complex that anchors the epithelial cells to the ECM protein laminin, which is formed by two subunits, α-DG and β-DG [3–5]. α-DG is located extracellularly, where it binds non-covalently to the extracellular domain of β-DG. Recent studies show that DG proteins may have a far wider role than just an epithelial cell–basal lamina

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interaction. It may play an important role in cancer development and progression. It has been shown that the expression of DG, in particular α-DG, is reduced or even lost in several cancer cell lines, as well as in a variety of cancers, for example prostate, breast, colon, cervical cancers, and gliomas. Moreover, reduced expression of α-DG may contribute to worsened prognosis in various cancers including breast, pancreas, and gliomas [6–9]. β-DG is a transmembrane protein with signaling and structural functions in its cytoplasmic region. It shows that in the majority of human cancers there is loss or weak expression of β-DG at both intercellular and basal cellular junctions [10, 11]. However, most studies on DG expression are performed on primary cancer tissues, little is known about alterations of DG in metastatic cancer sites.

DG expression is rarely studied in gastric cancer. Moon et al. [12] demonstrates that there is a possible correlation of α-DG with the biological behavior in gastric cancer cells, increment of α-DG expression may connect with poor prognosis among gastric cancer patients with liver metastasis. However, the role of β-DG is not indicated in his study. We hypothesize that both α-DG and β-DG may play a role in the oncogenesis and serve as a tumor suppressor gene in gastric cancer.

In the present study, expression of dystroglycan including α-DG and β-DG was analyzed by immunostaining in specimens of primary tumor, metastatic lymph node, distal metastatic site and their normal counterpart tissues, the relationship between DG expression and clinical outcome was evaluated, the results obtained suggest that both α-DG and β-DG is reduced with tumor progression, and reduced α-DG expression in primary tumor as compared to its normal tissue is associated with patient survival in gastric cancer.

Materials and Methods

Patients

Data were retrieved from the tumor registry at the department of surgical oncology, Sir Run Run Shaw hospital, Zhejiang University College of Medicine, Hangzhou, China. We screened gastric cancer patients with lymph node metastasis (mLN) and synchronous or metachronous distal metastases between July 1999 and March 2007, and twenty patients with complete data and tissue specimens were included in this study. Formalin fixed, paraffin embedded specimens including normal tissues adjacent to the tumor, paired primary tumor, metastatic lymph nodes and distant metastatic site were retrieved for this study from the archives of the department of pathology, and two experienced pathologists confirmed the histological diagnosis of each lesion. This study was approved by the institutional review board of Sir Run Run Shaw hospital. Prior to surgery, none of the patients received any anti-cancer treatment. Thirteen patients received postoperative chemotherapy but seven patients refused to receive chemotherapy. Patient clinical and histological characteristics were shown in Table 1.

Immunohistochemistry

Immunohistochemical analysis for expression of α-DG and β-DG was performed on formalin-fixed, paraffin-embedded surgical specimens. The slides were deparaffinized in xylene and rehydrated in gradient ethanol solutions. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 10 min. The slides were immersed in 10 mM citric buffer (pH 6.0) with heating for 15 min for antigen retrieval. Nonspecific binding was blocked by preincubation with 10% fetal calf serum in PBS with 0.01% sodium azide, and the slides were incubated in a humid chamber for 1 h with antibody against α-DG (mouse monoclonal, VIA41, Santa Cruz; 1:30) and β-DG (mouse monoclonal, 43DAGl/8D5, Novocastra; 1:200). The slides were incubated with the EnVision-HRP complex (undiluted, DAKO) for 60 min, then

Table 1 Clinical and pathological features of 20 gastric cancer patients with lymph node and distal metastasis

Clinicopathologic parameters	Number (percent)
Age (yr)*	55.4±2.9
Gender	
Male	14 (70%)
Female	6 (30%)
Tumor differentiation	
Differentiated	8 (40%)
Undifferentiated	12 (60%)
Location	
Cardia	5 (25%)
Corpus	8 (40%)
Antrum	6 (30%)
Whole stomach	1 (5%)
Tumor size (cm)*	6.1±0.3
Depth of invasion	
pT3	16 (70%)
pT4	4 (30%)
Distal metastatic site	
Liver	10 (50%)
Mesentery	8 (40%)
Ovary	1 (5%)
Pancreas	1 (5%)
Distal metastasis	
Synchronous	16 (80%)
Metachronous	4 (20%)

*represents Mean±SD

visualized with diaminobenzidine (DAKO Corp.) and counterstained with hematoxylin. For substitute negative controls, the primary antibody was replaced with phosphate buffered saline. Positive control was normal gastric tissue known to exhibit high expression of α-DG and β-DG.

The expression of the antibodies was assessed semi-quantitatively by estimating the percentage of tumor cells with positive cytoplasm staining on whole tumor slides. All the slides were examined and scored independently by two experienced pathologists to avoid subjective biases. Each slide was examined in its entirety under a light microscope, and initially a proportion score was assigned, which represented the estimated proportion of positive tumor cells (0, none; 1, 1~10%; 2, 11%~50%; 3, 51%~75%; and 4, >75%). Next, an intensity score was assigned, which represented the average intensity of the positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The total histologic score was expressed as a product of the intensity and area scores. Histologic score of 0 was defined as negative dystroglycan expression, whereas scores >2 were considered as dystroglycan expression [13].

Follow up

The patients were followed up until death or until the date of last follow-up of September 30, 2009. One patient had been lost to follow-up, and seventeen of 20 (85.0%) patients died during the follow-up period. The median follow-up interval was 25.7 months (range: 5.2–66.2 months).

Statistical Analysis

All statistical analyses were conducted using the statistical program SPSS 15.0 for Windows (SPSS, Chicago, IL, USA). Patient characteristics were analyzed using the 2-tailed chi-square test, comparison of DG expression was performed using independent *t*-test or paired *t*-test. Univariate analysis of patient survival was performed using Kaplan-Meier method. The survival curves were compared using the log-rank test. Multivariate analysis to identify the prognostic factors for survival was analyzed using the Cox proportional hazards regression model. The accepted level of significance was set as $p<0.05$.

Results

Changing Patterns of DG Expression in Primary Gastric Cancer and Metastatic Site

The expression of DG was evaluated by immunostaining. Examples for positive staining of DG expression were shown in Fig. 1. Immunostain of α-DG expression was detected in 19 (95%) out of 20 normal specimens, and positive expression of α-DG in the primary tumor, metastatic lymph node and distal metastatic site was detected in 70%, 25%, and 5% specimens, individually. Expression of α-DG in the metastatic specimens, including lymph node and distant metastasis site, was more reduced than that in the primary tumor specimens, and these differences were statistically significant (metastatic lymph node vs. primary tumor, $P<0.001$; distant metastatic site vs. primary tumor, $P<0.001$). α-DG expression in distant metastatic sites trend to have more reduction than that in metastatic lymph node, however, this difference did not reach significance ($P>0.05$).

Regarding β-DG, positive expression of β-DG in normal mucosa, primary tumor, metastatic lymph node and distal metastatic site was detected in 70%, 55%, 10% and 10% specimens, individually. β-DG expression in cancer cells was significantly reduced as compared to that in normal mucosa ($P<0.001$). In cancer specimens, more reduced expression of β-DG in the metastatic specimens was detected as compared to that in the primary tumor specimens, these differences were statistically significant (metastatic lymph node vs. primary tumor, $P=0.007$; distant metastasis site vs. primary tumor, $P=0.003$). The β-DG expression in the metastatic lymph node and distal metastatic site was similar in both groups ($P>0.05$).

Correlations Between DG Expression in Primary Cancer Site and Patient Survival

Changes of DG expression in gastric cancer tissues compared to normal tissues showed a connection with overall survival rather than absolute values in primary tumor tissues staining only. Accordingly, the difference in DG expression between paired tumors and normal tissues [DG (primary tumor-normal)] was calculated for gastric cancer patients with synchronous distal metastasis ($N=16$). For the expression of α-DG, patients with α-DG(primary tumor-normal)<0 showed poor survival ($p=0.002$)(Fig. 2a). Regarding β-DG, patients with β-DG (primary tumor-normal)<0 showed a trend toward a poor survival, however, this difference was not reach statistical significance ($p=0.079$)(Fig. 2b).

Correlations Between DG Expression in Metastatic Site and Patient Survival

To evaluate associations between differences in DG expression of primary tumor sites and metastatic sites and survival in gastric cancer patients with synchronous distal metastasis ($N=16$), both the DG (mLN- primary tumor) and

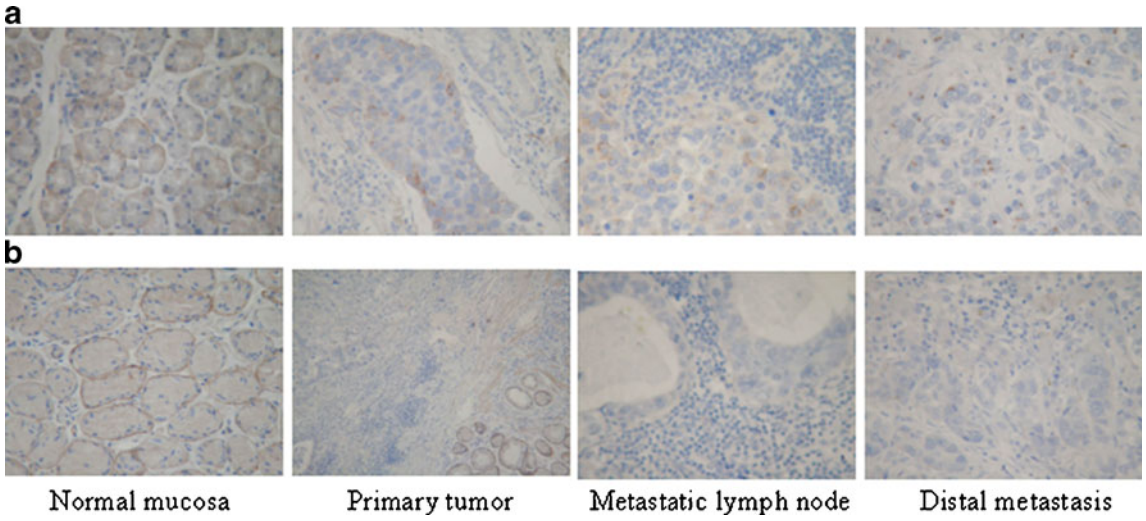

Fig. 1 Immunohistochemical staining of α-DG **a** and β-DG **b** in representative cases of gastric cancer. (original magnifications ×200)

DG (distal site-primary tumor) in tumors were calculated. For both α-DG and β-DG, patients with DG (mLN-primary tumor) <0 represented same survival as compared to DG (mLN-primary tumor)≥0 patients (α-DG, p=0.466; β-DG, p=0.322)(Fig. 3). Median survival of patients with DG (distal site -primary tumor) <0 was not significantly shorter than DG (distal site—primary tumor)≥0 patients (α-DG, p=0.385; β-DG, p=0.173) (Fig. 3).

Prognosticators for Patient Survival

In the present study, patients with gastric cancer and synchronous distal metastasis were selected for multivariate analysis, eight factors were entered into the Cox regression model including age, sex, tumor location, tumor diameter, differentiation, depth of invasion, α-DG (primary tumor-normal) and β-DG (primary tumor-normal). Although the total number of patients was small, α-DG (primary tumor-normal) was a prognosticator for patient survival (p=0.025), as shown in Table 2.

Discussion

DG is a transmembrane glycoprotein expressed in a wide variety of tissues, The functions of DG are complicated and related to cell-to-ECM communication, and the lost of cell-to-ECM communication may play an important role in cancer progression and metastasis [14]. Thus, it is of interest to investigate whether changes occur in the expression of DG during cancer tumorigenesis, DG is a recently focused adhesion molecule with a possible role in cancer development and progression. This is the first study to assess differences of DG expression between normal tissue, paired primary tumor, metastatic lymph node and distal metastatic site tissues, and to investigate the relationship between changing patterns of DG expression and patient survival in gastric cancer.

Recently, reduced expression of α-DG in cancer cells was observed as compared to its corresponding normal tissues, our result was consistent with the previous studies. In contrast, our unique finding was that expression of α-DG

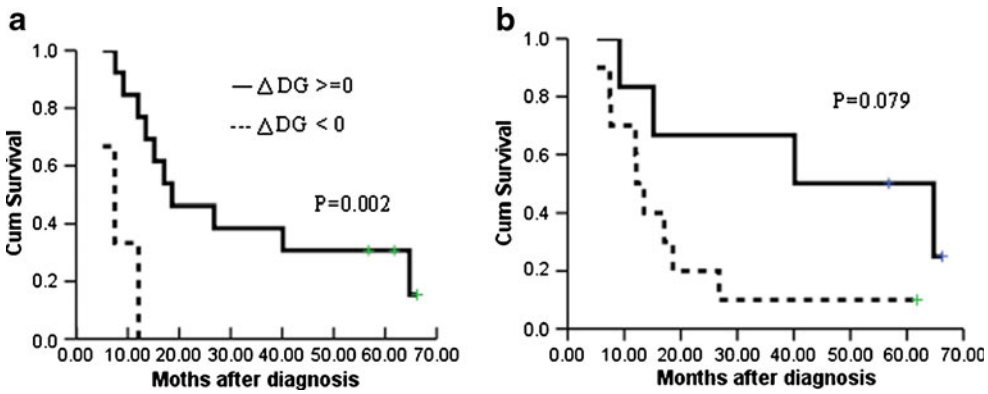

Fig. 2 Survival based on different expression of α-DG **a** and β-DG **b** between tumor and normal tissues in gastric cancer with synchronous distal metastasis

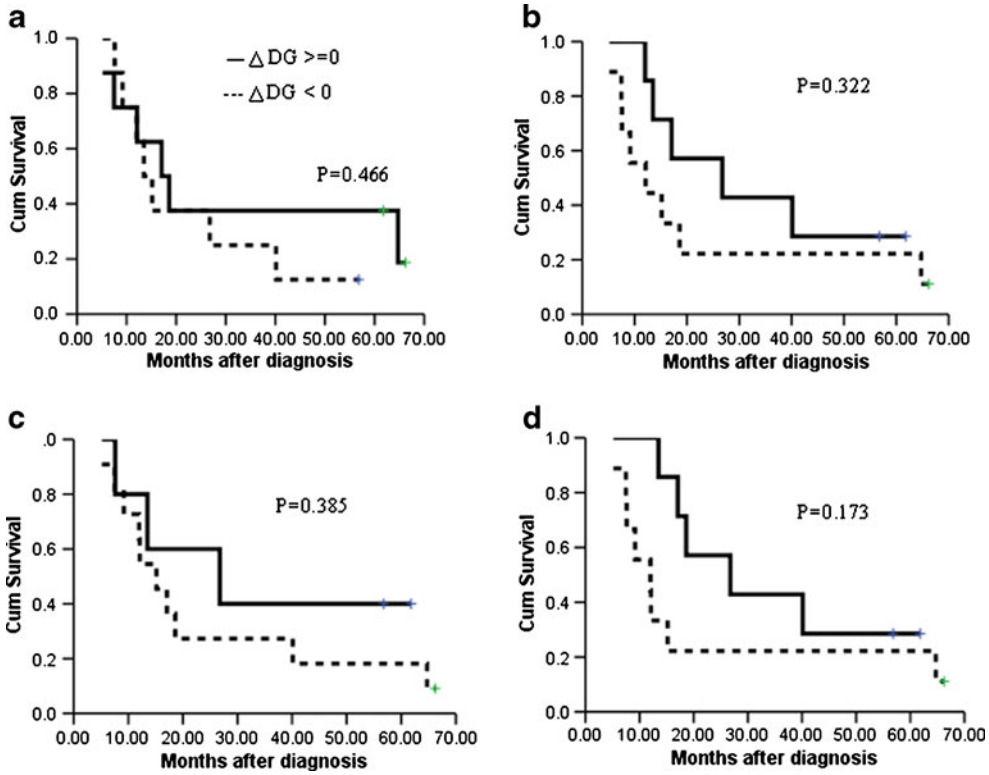

Fig. 3 Survival based on different expression of α-DG **a** and β-DG **b** between metastatic lymph node and primary tumor tissues and based on different expression of α-DG **c** and β-DG **d** between distal metastatic site and primary tumor tissues in gastric cancer with synchronous distal metastasis

in normal tissue, primary tumor site, metastatic site and distal site was gradually reduced, the difference of α-DG expression was statistically significant when compared between normal stomach tissues and primary tumor tissues, between primary tumor tissues and metastatic site including metastatic lymph node and distal metastatic site. However, when compared between metastatic lymph node and distal metastatic site, the difference of α-DG expression was not significant. As for the relationship between α-DG and patient prognosis, α-DG was correlated significantly with overall survival in a wide variety of tumors. Sgambato et al. reported that loss of α-DG expression is a frequent event in renal tumorigenesis and is an independent predictor of recurrence and overall survival for patients with renal cell carcinomas [15]. Similar results were found in breast cancer and colon cancer [8]. Moreover, aberrant expression of α-DG had been reported in human gastric cancer tissue, and low expression of α-DG showed poor overall survival in gastric cancers with liver metastasis [12]. We used the difference of α-DG expression between tumor and normal tissue to evaluate its relationship to survival, and our result showed reduced α-DG in primary tumor site as compared to normal site was associated with a shorter survival in gastric cancer patients, and multivariate analysis confirmed that reduced α-DG expression in primary tumor as compared to its normal counterpart was an independent prognostic indicator for survival. Our findings further strengthen the link between the expression of α-DG and gastric cancer and suggest that α-DG may play a role in gastric cancer progression and metastasis.

Table 2 Multivariate analysis of predicators for survival in patients with gastric cancer and synchronous distal metastasis

Variable	P value
Age (years) (≤ 55 vs. >55)	0.319
Sex (male vs. female)	0.107
Tumor location (upper vs. low and middle body)	0.069
Tumor size (cm) (< 6.0 vs. ≥ 6.0)	0.221
Differentiation (differentiated vs. undifferentiated)	0.077
Depth of invasion (T3 vs. T4)	0.099
$^{\Delta}$α-DG* (primary tumor-normal) (≥0 vs.<0)	0.025
$^{\Delta}$β-DG* (primary tumor-normal) (≥0 vs.<0)	0.099

*$^{\Delta}$ DG indicates the difference of DG expression

The α-DG and β-DG are both produced by a single DG gene [5]. β-DG is a transmembrane protein with signaling and structural functions in its cytoplasmic region. α-DG binds non-covalently to the extracellular domain of β-DG. Reduced or altered expression of β-DG has been demonstrated in colon cancer and breast cancer [7, 16]. However, there has been little work on the β-DG molecule in human gastric cancer, which may well be a functionally significant protein in development of gastric cancer. In the current study, changing patterns of β-DG in gastric cancer was evaluated. As the same as α-DG, gradually reduction of β-DG expression was observed in primary tumor, metastatic lymph node and distal metastatic site as compared to its

paired normal tissues. The difference of β-DG expression between each group was statistically significant except for groups between metastatic lymph node and distal metastatic site. Regarding the relationship between β-DG and patients survival, the difference of β-DG expression between tumor and normal tissues was not associated with patient survival, this result was the same as reported by Jiang XJ et al. [17], who demonstrated that the median survival time in patients with low expression of β-DG was not significantly different as compared to the patients with high expression of β-DG in pancreatic cancer.

In the present study, we observed that, for both α-DG and β-DG, the different expression of DG between primary tumor and paired metastatic lymph node, or between primary tumor and distal metastasis, was not associated with survival in gastric cancer with synchronous metastasis, and these results were not in accordance with previous study reported by Moon et al.[12], who found that higher α-DG expression in liver metastasis than in stomach tumors led to poor survival in gastric cancer with liver metastasis. One of the possible explanations for our results is we enrolled gastric cancer patients with multiple location of distal metastasis. The other is, the immunostain histoscore method was different and it may influence the results of DG expression in gastric cancer. Since these results are based on a small number of patient analyses, a large-scale analysis should be performed to confirm these results.

Based on our data, there were some weaknesses in the study. Firstly, because fresh tissue was not available, western blot for dystroglycan could not be performed, and these results were based on immunohistochemistry in paraffin embedded specimens. Secondly, our study has a very small number of patients. Although it is not possible to make definitive conclusions from these results, we can suggest a hypothesis that there is a trend to gradually reduction of DG expression in normal tissue, paired primary cancer and metastatic site, different expression of α-DG between tumor and normal tissue may be associated with prognosis in gastric cancer.

In conclusion, our results observed gradually reduction of DG expression during tumor progression, lower α-DG expression in tumors than in normal counterparts correlated with poor patient survival, whereas such a correlation was not found in the case of β-DG. Difference of α-DG between primary tumor and its normal counterparts is an independent prognostic factor in gastric cancer, these results point towards a more important role of α-DG in gastric cancer behavior, α-DG may not only link the extracellular matrix and the cytoskeleton but also play a role as a suppressor in gastric cancer, further experiments on the functional role of DG in gastric cancer will be necessary.

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