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RAPID COMMUNICATION

Conversion of cadherin isoforms in cultured human gastric carcinoma cells

Bing-Jing Wang, Zhi-Qian Zhang, Yang Ke

Bing-Jing Wang, Zhi-Qian Zhang, Yang Ke, Beijing Institute for Cancer Research and School of Oncology, Peking University, Beijing 100034, China
Supported by the National Natural Science Foundation of China, No.30370555 and No.30270658, National Major Basic Research Development Program No. G2000057002, and "211" project.
Correspondence to: Dr. Zhi-Qian Zhang, Department of Cell Biology, Beijing Institute for Cancer Research, Haidian District, Beijing 100036, China. zqzhang@public3.bta.net.cn
Telephone: +86-10-88196792 Fax: +86-10-88122437
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Abstract

AIM: To explore the expression of cadherin isoforms in cultured human gastric carcinoma cells and its regulation.

METHODS: The expressions of cell adhesion molecules (including E-cadherin, N-cadherin, α-catenin, β-catenin) and cadherin transcription factors including snail, slug and twist were determined by reverse transcriptase-polymerase chain reaction(RT-PCR), immunoblotting and immunofluorescence in SV40-immortalized human gastric cell line Ges-1 and human gastric cancer cell lines MGC-803, BGC-823 and SGC-7901.

RESULTS: All cell lines expressed N-cadherin, but not E-cadherin. N-cadherin immunofluorescence was detected at cell membranous adherents junctions where co-localization with immunofluorescent staining of inner surface adhesion proteins α- and β-catenins was observed. The transformed Ges-1 and gastric cancer cell lines all expressed transcription factors (snail, slug and twist) which inhibited the expression of E-cadherin and triggered epithelial-mesenchymal transformation.

CONCLUSION: Cadherin isoforms can change from E-cadherin to N-cadherin in transformed human gastric cancer cells, which is associated with intracellular events of stomach carcinogenesis and high expression of corresponding transcription factors.

Key words: E-cadherin; N-cadherin; Transcription factor; Gastric cancer

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INTRODUCTION

Cadherins are a family of transmembranous glycoproteins responsible for calcium-dependent intercellular adhesion, and play a dual role as adhesion ligands and receptors in cell adherents junctions (AJs). They are divided into more than ten subclasses, which are distinct in immunologenic specificities and tissue distribution. AJs are cell-to-cell adhesion sites where classic cadherins, cytoplasmic catenins (α-, β-, γ-) and actin-based cytoskeleton are assembled. Such protein complex plays a role as a functional unit in mediating cell adhesion and signal transduction and is involved in regulation of cell recognition, migration and cell sorting behavior [2, 3]. E-cadherin and N-cadherin, members of cadherin superfamily, are the most popular adhesion receptors in tissues. E-cadherin is predominantly expressed in epithelia, and loss of E-cadherin expression is a major characteristic of highly invasive and metastatic cancer [4], while re-expression of E-cadherin results in reversion of metastatic phenotypes to benign phenotypes. E-cadherin has been known as a tumor suppressor against invasion and metastasis of tumors [5]. N-cadherin is expressed in mesenchymal cells, but recent study demonstrated that N-cadherin is associated with an increased invasive potential of cancer [6]. Forced expression of N-cadherin exerts a dominant effect over E-cadherin function in breast cancer cells [7].

In early embryonic development, loss of E-cadherin accompanying the acquisition of fibroblastic phenotype occurs through a mechanism called epithelial-mesenchymal transition (EMT). This is an essential event during gastrulation and neural crest formation [8] as well as in early invasion and metastasis of carcinoma cells [9]. Multiple mechanisms can inactivate E-cadherin in cancer cells, such as gene mutation, promoter hypermethylation, chromatin rearrangement, and transcriptional repressors. Several developmentally important genes inducing EMT act as E-cadherin repressors. In vertebrates, snail and its closely related gene slug bind directly to E-boxes in the *E-cadherin* promoter and repress *E-cadherin* expression directly in mouse and human invasive carcinoma cells [10]. A further molecule known to trigger EMT is twist, which is possibly involved in E-cadherin and N-cadherin conversion during

Table 1 PCR Primers used in experiments

Genes	Primers (sense/anti-sense)
E-cadherin (361bp)	5′-TCCATTTCTTGGTCTACGCC-3′ 5′-TTTGTCCTACCGACTTCCAC-3′
N-cadherin (373bp)	5′-GTGCCATTAGCCAAGGGAATTCAGC-3′ 5′- GGAGGATACTCACCTTGTCCTTGCG-3′
Snail(377bp)	5′-CTGCAGGACTCTAATCCAG-3′ 5′- CGAGAGACTCCGGTTCCTA-3′
slug(410bp)	5′-AGCGAACTGGACACACATAC-3′ 5′-TCTAGACTGGGCATCGCAG-3′
twist(612bp)	5′-GCAAGCTTAGAGATGATGCAGGACG-3′ 5′-GACTCGAGGTGGGACGCGGACATGGA-3′
GAPDH (451bp)	5′-ACCACAGTCCATGCCATCAC-3 5′-ATGTCGTTGTCCCACCACCT-3′

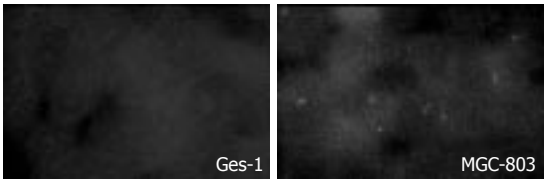

Figure 1 Expression of E-cadherin in SV40-immortalized Ges-1 and gastric cancer cell lines. Negative staining of E-cadherin in Ges-1 and MGC-803 was shown on immunofluorescent micrographs.

EMT [11]. In the present study, we chose a transformed human gastric cell line as control and three human gastric cancer cell lines to detect the expression of E-cadherin and N-cadherin subtypes and their associated adhesion protein (α- and β-catenins) related to changes of transcription factors (snail, slug and twist), and to analyze the role of snail and other EMT regulators in down-regulating E-cadherin in gastric cancer cells.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cell lines MGC-803, BGC-823 and SGC-7901 were cultured in RPMI1640 medium (GIBCO/BRL) containing 10% fetal bovine serum. Ges-1, a non-tumorigenic but immortalized human gastric cell line, was obtained by SV40 viral transformation and cultured in RPMI1640 medium supplemented with 15% fetal bovine serum [12].

Immunoblotting experiments

Cells of logarithmic growth potential treated with RIPA lysis buffer (50mmol/L Tris-HCl pH 7.4, 150mmol/L NaCl, 1% NP40, 50mmol/L NaF, 0.1mmol/L Na_3VO_4, 1mmol/L phenylmethylsulfonyl fluoride, 10μg/mL leupeptin, 0.1% SDS, 0.5% deoxycholic acid, 50mmol/L HEPES, 10mmol/L EDTA, 50mmol/L sodium pyrophosphate, 50mmol/L ammonium molybdate) were used to determine the total protein concentration according to Bradford protein quantification method. Samples of equal amount were loaded onto 10% SDS-PAGE gel and electrophoretically transferred to PVDF membrane, blocked with 5% non-fat milk/TTBS, reacted with primary antibody, washed with TTBS and labeled with goat and anti-mouse or rabbit secondary antibody. The primary antibodies used were mouse anti-N-cadherin monoclonal antibody, rabbit anti-E-cadherin polyclonal antibody, rabbit anti-α- and β-catenin polyclonal antibody (Sigma). Protein signals were detected using ECL chemiluminescence reagent (Amersham Phamacia).

Indirect immunofluorescent staining

Cells growing on coverslips were fixed in 2% formaldehyde for 3 min and extracted 3 times (10 min each) with 0.5% Triton X-100. The cell samples were incubated with primary antibodies (described above) at 37°C for 1 h, washed with PBS containing 0.5% Triton X-100 and then reacted with FITC- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at 37°C for 1 h. After washed, the samples were rinsed in 0.9% NaCl, stained with 1μg/L 4, 6-diamidino-2-phenylindole (DAP; Sigma) and mounted in 60% glycerol/PBS. Olympus BH2 fluorescent microscope equipped with FITC- and rhodamine- channel filter systems was used to observe distribution of different proteins in the same fields. Kodak TMAX-400 black and white films were used for photographs.

RT-PCR

Total RNA was extracted from cells of logarithmic growth potential with Trizol (Invitrogen) as described, then suspended in 50μL RNase-free water and quantified. Reverse-transcription was performed in a final volume of 20μL using superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions under the following conditions:1mmol/L dNTP, 50ng Oligo-dT_{15} and 3μg total RNA. The reaction was performed at 50°C for 45 min, followed by inactivation of the enzyme at 70°C for 15 min. The sequences of the specific primers are listed in Table 1. Forty cycles of PCR amplification were performed at 94°C for 1 min, at 58°C for 1 min, at 72°C for 1 min. PCR products were identified by running on 1.2% agarose gel.

RESULTS

E-cadherin expression lost in SV40-immortalized Ges-1 and gastric cancer cell lines

The three gastric cancer cell lines MGC-803, BGC-823, and SGC-7901 were negative for E-cadherin expression as demonstrated by RT-PCR, immunoblotting and immunofluorescent staining. The SV40-transformed gastric epithelial cell line Ges-1, non-tumorigenic in nude mice with normal cell morphology, was also negative for E-cadherin expression at mRNA and protein levels (Figure 1).

N-cadherin expression in SV40-immortalized Ges-1 and gastric cancer cell lines

In order to understand the formation of adherens junctions in these E-cadherin negative cell lines, N-cadherin expression was detected by RT-PCR, immunoblotting and

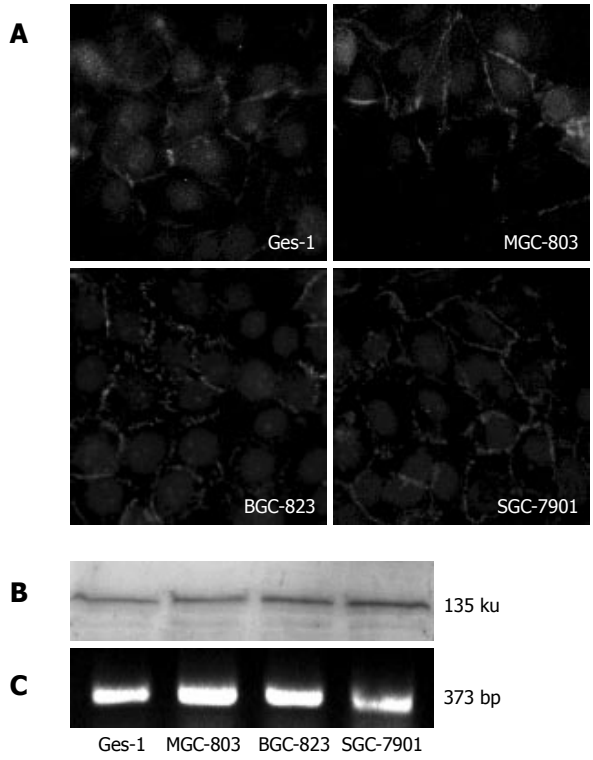

Figure 2 Expression of N-cadherin in SV40-immortalized Ges-1 and gastric cancer cell lines. **A**: Distribution of N-cadherin fluorescence on cell membranes in Ges-1 and gastric cancer cell lines; **B**: A band of 135 ku N-cadherin protein detected by Western blotting of N-cadherin; **C**: N-cadherin mRNA expression shown by RT-PCR.

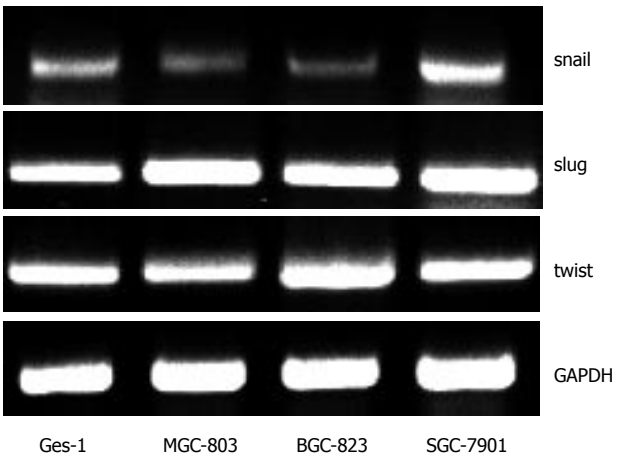

Figure 4 Expression of E-cadherin transcription repressors (snail, slug) and N-cadherin activator (twist) in Ges-1 and gastric cancer cell lines.

immunofluorescent staining (Figure 2). N-cadherin mRNA was found in all these E-cadherin negative cell lines (SV40-transformed Ges-1, BGC-823, MGC-803, and SGC-7901). In consistence with N-cadherin mRNA expression, a band of 135 ku N-cadherin protein was also shown in these cell lines as detected by Western blotting of N-cadherin. N-cadherin fluorescence was distributed at cell-cell contact membrane regions, suggesting that these cells established adherents junctions through N-cadherin rather than E-cadherin. Alteration of cadherin isoforms from E-cadherin to N-cadherin occurred in the process of carcinogenesis.

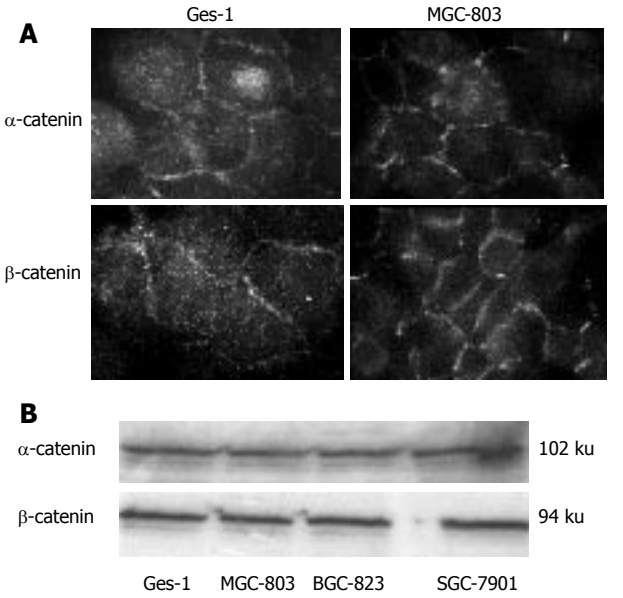

Figure 3 Expression of α- and β-catenins in Ges-1 and gastric cancer cell lines. **A:** Distribution of α- and β-catenin fluorescence on cell membranes and cell-cell borders in Ges-1 and MGC-803; **B:** Reactive bands at 102 ku and 94 ku of α- and β-catenins in Ges-1 and cancer cell lines.

Expression of α- and β-catenins in SV40-immortalized Ges-1 and gastric cancer cell lines

Immunoblotting of α- and β-catenins showed reactive bands at 102 ku and 94 ku respectively in Ges-1 and gastric cancer cell lines. The fluorescence of α- and β-catenins was found at the cell-cell contact borders irrespective of transformed cells or cancer cells (Figure 3). The colocalization of both cadherin and catenins indicated the formation of cadherin/catenin complex at adherents junctions.

Expression of E-cadherin transcription repressor and N-cadherin activator in Ges-1 and gastric cancer cell lines

The transcription factors (snail, slug and twist) could repress the expression of E-cadherin and induce the expression of N-cadherin. Thus, we examined the expression of these transcription factors in these cell lines with alteration of E-cadherin to N-cadherin by RT-PCR. They were all expressed in Ges-1 and three cancer cell lines, but their expression levels varied (Figure 4).

DISCUSSION

It is well known that E-cadherin is an adhesion receptor of normal epithelial cells that binds to cytoplasmic adhesion proteins to form E-cadherin/catenin complex as an anchorage site for actin-based cytoskeleton. The E-cadherin/catenin/cytoskeleton complex plays an important role in the maintenance of structural and functional stability of epithelial tissues. Loss of E-cadherin expression or function in epithelial carcinoma has been considered as a primary reason for disruption of cell-cell contacts. However, there is evidence that other cadherins, such as N-cadherin, are up-regulated in cancer cell lines [13]. N-cadherin is present in invasive cancer cell lines and its exogenous expression in tumor cells increases the ability

of invasion and metastasis of tumor cells. Changes in the expression of cadherins occur in the early embryonic development during gastrulation and neural crest formation. The loss of E-cadherin with the acquisition of fibroblastic phenotype occurs through a mechanism called epithelial-mesenchymal transition (EMT) during early invasion and metastasis of carcinoma cells. To investigate the occurrence of cadherin conversion in gastric carcinoma, we determined the expression of E-cadherin and N-cadherin in transformed human gastric cell line and three gastric cancer cell lines. The results demonstrated that three human gastric cancer cell lines were all negative for E-cadherin immunofluorescent staining and immunoblotting, which is in agreement with the reported data, suggesting that E-cadherin expression is inhibited in gastric cancer cells [14]. Meanwhile, our finding is in accordance with the report that loss of E-cadherin is a key step in the progression from benign adenoma to malignant carcinoma *in vivo* [15]. However, in our study, N-cadherin was expressed in all these E-cadherin negative cell lines and N-cadherin fluorescence was distributed at cell-cell contact membrane regions, suggesting that these cells are established at the adherents junctions through N-cadherin rather than E-cadherin. Apparently, alteration of cadherin isoforms from E-cadherin to N-cadherin occurs in the process of carcinogenesis. SV40-transformed gastric epithelial cell line Ges-1 characterized by immortalization *in vitro* and normal cell morphology, was also negative for E-cadherin expression at mRNA and protein levels, suggesting that inhibition of E-cadherin expression may be an early cellular event in the process of cell malignancy.

The interaction between cytoplasmic cadherins and catenins is critical for the formation of stable and functional AJs. The mutation or homozygous deletion of catenin gene leads to loss of cell-cell adhesion. Moreover, β-catenin can act as a transcrption factor in nuclei as an activator of LEF/TCF family of the DNA-binding proteins, which is activated by the Wnt signal pathway [16]. We therefore investigated the expression of α- and β-catenins in the same cell lines. Immunoblotting and immunofluorescence staining showed positive expressions of α- and β-catenins in transformed Ges-1 and three cancer cell lines. Meanwhile, we did not find the relocalization of β-catenin in nuclei, suggesting that cadherin/catenin complex is formed at adherents junctions. In gastric cancer cells, the formation of N-cadherin/catenin complex at AJs after conversion of cadherin isoforms may imply that the mediated adhesion signals are needed for remote metastasis of cancer cell mass.

Multiple mechanisms can inactivate E-cadherin in cancer cells. Recent reports have highlighted the role of EMT regulator, snail, a strong repressor of E-cadherin gene expression in cancer cell lines [17, 18]. Snail is a zinc finger transcription factor which directly represses E-cadherin expression by binding to E-box DNA-binding sequence CAGGTG and is required for mesoderm differentiation and neural crest formation during embryonic development. In human carcinoma and melanoma, the expression of snail correlates with the absence of E-cadherin expression [19-21]. Forced expression of snail in cultured epithelial cells represses the E-cadherin promoter and increases invasion and matrix metalloproteinase-2 expression [22]. Slug, which also represses the E-cadherin promoter, can likewise induce EMT, increase cell motility and invasion and confer tumorigenesis [23]. Twist is originally identified in *Drosophila* as a protein involved in establishing dorso–ventral polarity and in epithelial–mesenchymal transition. Recent findings indicate that twist is both an E-cadherin repressor and an EMT inducer [24]. Furthermore, the findings implicate that twist is involved in tumor cell intravasation and in tumor metastasis. In the present study, strong expressions of snail, slug and twist were observed in the transformed gastric cell line Ges-1 and three gastric carcinoma cell lines. Meanwhile, these cell lines were all negative for E-cadherin expression but positive for N-cadherin expression.

In conclusion, EMT regulators play a critical role in human gastric carcinoma. The transcription factors (snail, slug and twist) are correlated with the alteration of cadherin isoforms in human gastric cancer cells. However, the mechanism of how the transcription factors regulate the alteration of cadherin isoforms remains to be elucidated.

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