Expression of N-cadherin in esophageal squamous cell carcinoma and silencing expression of N-cadherin using RNA interference on invasiveness of EC9706 cells

Ke Li, Xin Wang, Wei He, Na Lin and Qing-Xia Fan*

Department of Oncology; the First Affiliated Hospital; Zhengzhou University; Zhengzhou, Henan P.R. China

Key words: esophageal neoplasms, squamous cell carcinoma, RNA interference, N-cadherin, EC9706 cells

Background and Objective: E- and N-cadherin are calcium-dependent cell adhesion molecules that mediate cell–cell adhesion and also modulate cell migration and tumor invasion. It has been suggested that, unlike E-cadherin, N-cadherin may promote invasion and metastasis of carcinoma. This study was to explore the correlation of E-cadherin and N-cadherin expression to clinicopathologic features of esophageal squamous cell carcinoma (ESCC), and to investigate the effect of silencing N-cadherin expression by RNA interference (RNAi) on the invasiveness of EC9706 cells. Methods: PV immunohistochemistry was used to detect the expression of E-cadherin and N-cadherin in 62 specimens of normal esophageal epithelium, 31 specimens of adjacent atypical hyperplasia epithelium and 62 specimens of ESCC. N-cadherin siRNA was transfected into EC9706 cells, and the effect of RNAi was assessed by RT-PCR and Western blot. The invasiveness of EC9706 cells was determined by Transwell chamber assay. Results: The positive rates of E-cadherin and N-cadherin were 95.2% and 29.0% in normal esophageal epithelium, 71.0% and 61.3% in adjacent atypical hyperplasia epithelium, 40.3% and 75.8% in ESCC. The negativity of E-cadherin and positivity of N-cadherin were correlated to invasion, differentiation, and lymph node metastasis of ESCC (p < 0.05). E-cadherin expression was negatively correlated to N-cadherin expression in ESCC (γ = -0.534, p < 0.05). N-cadherin RNAi significantly inhibited N-cadherin expression in EC9706 cells, and decreased the number of EC9706 cells that invaded through the basement membrane from (123.40 ± 8.23) to (49.60 ± 6.80) (p < 0.05). Conclusions: Down-regulation of E-cadherin and up-regulation of N-cadherin may be involved in the genesis of ESCC. Silencing N-cadherin using RNA interference could inhibit the invasiveness of EC9706 cells in vitro.

Invasion and metastasis of malignant tumor seriously threaten the life of cancer patients. The cadherin family is closely related to the invasion and metastasis of tumors, among which E-cadherin and N-cadherin are the two most widely distributed members. Many reports in China reveal that low-expression or un-expression of E-cadherin is related with infiltration and metastasis of esophageal squamous cell carcinoma (ESCC).1,2 Recent studies show increased N-cadherin expression in prostatic carcinoma and breast cancer, which plays a more important role than decreased expression of E-cadherin in inducing infiltration and metastasis of tumor cells.3,4 N-cadherin is crucial for the adhesion of tumor epithelial cells to vascular smooth muscle cells and pericytes expressing N-cadherin. During tumor formation, the expression of N-cadherin contributes to vasoformation and immigration of epithelial-mesenchymal cells, so that the tumor cells are more invasive and easier to metastase.5

In the present study, expressions of E-cadherin and N-cadherin proteins in ESCC, adjacent atypical hyperplastic epithelium and normal esophageal epithelium were detected, and their relationship with clinicopathological factors of ESCC were analyzed. In addition, down-regulation of N-cadherin on invasiveness of human esophageal carcinoma cell line EC9706 by retrovirus mediated RNA interference was also observed. Meanwhile, the changes in gene expression of E-cadherin and MMP-9 before and after RNA interference were detected.

Materials and Methods

Materials. pEGFP-MSCVneo plasmid and pMSCVneo/N-cadherin plasmid were kindly provided by Dr. Ma from Beijing Union Medical University. The size of pMSCVneo/N-cadherin plasmid was 7.5 kb, containing the EGFP gene sequence, the U6 promoter sequence and multiple clone sites. Human esophageal carcinoma cell line EC9706 was provided by Chinese Academy of Science. Packaging cell line PT67 was purchased from Clontech, USA. Primary antibodies (mouse anti-human monoclonal antibodies) for E-cadherin, N-cadherin and MMP-9 were purchased from abcam, USA. Matrigel was purchased from BD, USA. Lipofectamine™2000 was purchased from Invitrogen, USA. Primers for E-cadherin,
N-cadherin and inner control β-actin were synthesized by Shanghai Sangong Bioengineering Ltd. Transwell chamber was purchased from Costar, USA.

Methods. Measurements of E-cadherin and N-cadherin expressions in ESCC. (1) Specimen preparation and treatments: Sixty-two excised specimens of esophageal carcinoma were taken from Anyang Cancer Hospital, Henan province between February 26, 2006 and March 16, 2006. There were 36 males and 26 females, aged 38–75 years old. All cases were confirmed by pathological examination before the surgery. None of the patients had history of chemotherapy, radiotherapy and immunotherapy. (2) Immunohistochemical staining: Expressions of E-cadherin and N-cadherin in ESCC were detected by immunohistochemistry. Sixty-two specimens were collected from non-neoplastic tumors, tissues within 3 cm of the cancer colli, and distal normal mucosa. Atypical hyperplasia was found in 31 para-cancerous tissues by H&E staining. Specimens were fixed in 40 g/L paraformaldehyde and then subjected to routine dehydration, followed by paraffin imbedding to obtain serial sections of 4–6 μm thick. Sixty-two cases were all confirmed as squamous cell carcinoma by histological examination, among which 15 cases were grade I, 25 cases were grade II, and 22 cases were grade III. In addition, 20 cases were complicated with nodal metastasis, while the other 42 cases were not. Seven cases were found infiltrated to submucosa or to the superficial muscular layer, while 55 cases were found infiltrated to the deep muscular layer or mantle layer. The working concentrations of mouse anti-human E-cadherin and N-cadherin monoclonal antibodies were 1:100 and 1:110, respectively. The two-step immunohistochemical kit (PV-9000) and DAB enhancer were also used. Procedures were strictly performed according to the manufacture's instructions. PBS was used to replace the primary antibody as negative control. (3) Judgment of the results: Brown yellow particles in the cell membrane/cytoplasm were considered as positive staining. E-cadherin was mainly distributed in the cell membrane, while N-cadherin was mainly in the cytoplasm. Scoring was made according to the staining intensity and the ratio of positive cells. The percentage of positive cells < 30% was scored 1, 30–60% was scored 2, and > 60% was scored 3. As for the staining intensity, no staining was recorded 0 (negative), pale yellow was recorded 1 (weakly positive), brown yellow was recorded 2 (positive), and brown was recorded 3 (intensive positive). The product of the two scores determined the final score values: 0-1 was regarded as negative, while ≥ 2 was regarded as positive.6 (4) Statistical analysis: χ2 test was performed using SPSS 11.0 statistical software. Correlation test was performed using the Spearman rank correlation test. The significance level α = 0.05 was adopted.

Silencing of N-cadherin gene expression in EC9706 cells using retrovirus mediated RNAi: (1) Transfection of PT67 packaging cells, screening and amplification of G-418 resistant clones: PT67 packaging cells were plated onto six-well culture plates and transfected by lipofectamine 2000 when they reached 90–95% confluence. The cells were divided into four groups: control group, lipofectamine group, pEGFP-MSCVneo plasmid group and pMSCVneo/N-cadherin plasmid group.

G418 was used to screen and amplify resistant clones. The initial concentration of G418 was 1000 mg/L, which was obtained from the preliminary experiments, then the concentration was decreased to 300 mg/L 10–15 days later, and was maintained for another 10–15 days. Transfected cells were cultured under an inverted fluorescent microscope at 488 nm for the expression of green fluorescent protein every 3–4 days. (2) Detection of retrovirus titer: Two sharply defined clones were chosen from G418-resistant pEGFP-MSCVneo and pMSCVneo/N-cadherin clones respectively, and cultured in G418-free DMEM medium for 48 h. Supernatant was collected and filtered through a 0.45 μm sterilized filter membrane. Virus supernatant (1 mL) was mixed with 2 mL medium containing 10% fetal bovine serum (FBS). Polybrene was added to a final concentration of 8 mg/L. The resulted medium was added to NIH3T3 cells, and incubated for 3–5 h. Then the serum was removed and fresh medium was added. G418 (600 mg/L) was added for screening 24 h later. Untransfected NIH3T3 cells were used as control. The clone number in each flask was calculated two weeks later and the virus titer was calculated using the following formula: G418 resistant cfu/L = clone number / [volume of the virus stock solution (L) x replication factor x dilution]. Supernatant with the maximum virus titer was selected for further experiments. (3) Transfection of EC9706: EC9706 cells were plated into six-well culture plates and infected with virus after reaching 90–95% confluence. Cells were divided into three groups, the untreated group, pEGFP-MSCVneo group and pMSCVneo/N-cadherin group. EC9706 cell clones with G418 resistance were screened and maintained in the medium containing G418 (300 mg/L). (4) RT-PCR: RT-PCR was performed to detect mRNA expression of E-cadherin, N-cadherin and MMP-9 in the untreated group, pEGFP-MSCVneo group, pMSCVneo/N-cadherin group and breast cancer cell line 435 (positive control). β-actin was used as an internal control. Its upstream and downstream primers were 5'-CAT CCT GCG TCT GGA CCT-3' and 5'-TCA GGA GGA GCA ATG ATC TTG-3', yielding a product of 480 bp. The primers for E-cadherin were (upstream) 5'-GCT GCT TCT GCT TCT TCG-3' and (downstream) 5'-CCT CCT CCT TCT TCA TCA TAG-3', with a product of 104 bp. The primers for N-cadherin were (upstream) 5'-CAA CTT GCC AGA AAA CTC CAG G-3' and (downstream) 5'-ATG AAA CCG GCC GGC TAT CTC CTC-3', with a product of 204 bp. The primers for MMP-9 were (upstream) 5'-TGG AGT CAC TGT ACA CCC TC-3' and (upstream) 5'-GGG ACA TCC GCT AAA CTC CAG G-3', and the product size was 400 bp. Reaction parameters were as follows: pre-denaturation at 94°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 50 s, and 72°C for 60 s; followed by a final extension at 72°C for 7 min. Amplified PCR products were subjected to 2% agarose gel electrophoresis and observed under an ultraviolet lamp. Gray scale images of the bands were scanned and analyzed by the IBAS 2.0 true color image analyzer from Kontron (Germany). (5) Detection of E-cadherin, N-cadherin, and MMP-9 proteins by western blot: Cells from untreated group, pEGFP-MSCVneo group, pMSCVneo/N-cadherin group, and the positive control group were digested and washed by ice cold PBS for three times. Then lysis buffer was added and the cells were lysed on ice for 30 min, followed by centrifugation at 12,000 g for 5 min to remove cell debris. Protein samples (50 μg) were mixed with the loading buffer and boiled for 5 min, after which the samples were separated by 10% SDS-PAGE electrophoresis and transferred on a nitrocellulose membrane. The nitrocellulose membrane was blocked in TBST containing 5% defatted milk at room temperature for 1 h, followed by incubation with E-cadherin, N-cadherin and MMP-9 mouse anti-human monoclonal antibodies (1:500), and rabbit and
Expression of N-cadherin in esophageal squamous cell carcinoma and silencing expression of N-cadherin using RNA interference on invasiveness of EC9706 cells

mouse IgG-HRP secondary antibodies, successively. The bands were detected with the ECL chemoluminescence reagent, scanned and analyzed by the IBAS2.0 true color image analyzer from Kontron (Germany). (6) Transwell chamber assay: Cells from the untreated group, pEGFP-MSCVneo group and pMSCVneo/N-cadherin group were harvested and suspended in FBS free DMEM to prepare single cell suspension. Cell density was adjusted to $5 \times 10^5$ cell/mL. Matrigel was washed with serum free culture medium once, and 100 µl of cell suspension was added into each well. Condition medium of 500 µl containing 20% FBS was added to the lower chamber and the cells were cultured at 37°C for 20–24 h. Liquid in the upper chamber was removed and the upper chamber was taken out carefully. Non-permeating cells on the membrane were wiped out with a wet cotton swab. Permeating cells were fixed with 70% methanol for 45 min and stained with haematoxylin for 5 min. The staining was terminated by washing with flowing water. Cells migrated to the membrane were observed under a light microscope. The total number of cells in five visual fields (100-fold) of each well was counted, and the mean was calculated. The number of permeating cells was chosen as the index to evaluate the infiltration capacity of tumor cells. (7) Statistical analyses. All experimental data were statistically analyzed using SPSS11.0 software package. Comparison of means among multiple groups was performed with one-factor analysis of variance. A value of less than 0.05 was considered significant.

Results

Expression of E-cadherin and N-cadherin in ESCC. Expressions of E-cadherin and N-cadherin, and their relationship with clinicobiological behaviors of ESCC. Positive signals of E-cadherin were mainly located on the membrane of normal esophageal squamous cells, and were also slightly expressed in the cytoplasm as yellow or yellow brown particles (Fig. 1A and B). The positive rate of E-cadherin was decreased in order of normal esophageal mucous membrane, adjacent atypical hyperplasia and cancer tissues, which was 95.2% (59/62), 71.0% (22/31) and 40.3% (25/62) respectively (p < 0.05). Positive signals of N-cadherin were located in the cytoplasm as yellow or yellow brown particles (Fig. 1C and D). Its positive rate was increased in order of normal esophageal mucous membrane, adjacent atypical hyperplasia and cancer tissues, which was 29.0% (18/62), 61.3% (19/31) and 75.8% (47/62) respectively (p < 0.05). A summary of expressions of E-cadherin and N-cadherin in different tissues as well as their relationship with differentiation degree, invasion and metastasis of ESCC is listed in Table 1.

Correlation of E-cadherin and N-cadherin expression. Twelve out of 25 E-cadherin positive cases were positive for N-cadhein, and two out of 37 E-cadherin negative cases were negative for N-cadherin. The result of the spearman rank correlation test showed that the expression of E-cadherin was negatively correlated with that of N-cadherin ($\gamma = -0.534$, p < 0.05).
Expression of N-cadherin in esophageal squamous cell carcinoma and silencing expression of N-cadherin using RNA interference on invasiveness of EC9706 cells

Effects of N-cadherin RNAi in EC9706 cells. Transfection of packaging cell PT67, screening and amplification of positive clones. Cells were observed under a fluorescent microscope at 488 nm 24 h after transfection. Bright green fluorescence was observed in the cytoplasm and nucleus of both the pEGFP-MSCVneo plasmid group and pMSCVneo/N-cadherin plasmid group, but was not observed in the control group or liposome group. Cells in the control group and liposome group died 20–30 days after transfection, thereby stably transfected PT67 cells were obtained (Fig. 2).

Determination of retrovirus titer. The virus titer of PT67 cell clone 1 and clone 2 in the pEGFP-MSCVneo plasmid group was 6 x 10^7 cfu/L and 1 x 10^7 cfu/L, respectively, while that in the pMSCVneo/N-cadherin plasmid group was 2 x 10^6 cfu/L and 8 x 10^6 cfu/L, respectively. Therefore, supernatant of PT67 cell clone 1 from the pEGFP-MSCVneo plasmid group, and that of PT67 cell clone 2 from the pMSCVneo/N-cadherin plasmid group were selected for transfection into EC9706.

Transfection of EC9706 with N-cadherin RNAi. All cells in the control group died 20-30 days after infection, while stably transfected EC9706 cells were obtained (Fig. 3).

Expression of E-cadherin, N-cadherin, and MMP-9 mRNA before and after transfection of EC9706 cells with N-cadherin RNAi (Fig. 4). (1) mRNA expression of E-cadherin: Weak bands at 104bp corresponding to E-cadherin were observed in positive control group (0.313 ± 0.011), untreated group (0.32 ± 0.021), pEGFP-MSCVneo group (0.330 ± 0.065) and pMSCVneo/N-cadherin group (0.328 ± 0.006). The differences were not statistically significant (p > 0.05). (2) mRNA expression of N-cadherin: Expressions of N-cadherin mRNA (204bp) were significantly higher in the positive control group (0.663 ± 0.016), untreated group (0.661 ± 0.023), pEGFP-MSCVneo group (0.330 ± 0.065) and pMSCVneo/N-cadherin group (0.328 ± 0.006). The differences were not statistically significant (p > 0.05). (3)mRNA expression of MMP-9: Expressions of MMP-9 (~400bp) were significantly higher in the positive control group (0.763 ± 0.016), untreated group (0.741 ±...
Expression of N-cadherin in esophageal squamous cell carcinoma and silencing expression of N-cadherin using RNA interference on invasiveness of EC9706 cells

Discussion

Expressions of E-cadherin and N-cadherin, and their relationship with clinicobiological behaviors of ESCC. The cadherin family, consisting of a serial of single strand transmembrane glycoproteins with similar structures and functions, is mainly responsible for mediating intercellular adhesion of Ca\(^{2+}\) dependent homotypes. It can either be a receptor or a ligand to bind with each other in a homophilic way. It plays an important role in cell recognition, migration, sorting behavior, and maintaining the normal tissue structure, as well as participates in regulating histogenesis and morphodifferentiation.\(^7\)

Currently, it is believed that E-cadherin plays its role as an inhibitory factor of tumor metastasis. Deficiency in protein expression of E-cadherin is closely related to the generation and development of lung cancer, liver cancer, prostatic carcinoma and breast cancer, and so on.\(^8,9\) During the progression of human lung cancer, E-cadherin gene is decreased, particularly in patients with lymph node metastasis. E-cadherin expression and survival rate are gradually decreased following the progression of the TNM stage.\(^10\)

Recent studies\(^3,4\) show that the expression of N-cadherin is increased in breast cancer and prostatic carcinoma. N-cadherin plays a more critical role in inducing tumor infiltration and metastasis than E-cadherin. Utsuki et al.\(^11\) claim that the increased expression of N-cadherin in brain glioblastoma and astrocytoma is related to the pathological grading of tumors, accompanied by a decease of E-cadherin. Makagiansar et al.\(^12\) find that N-cadherin can increase the adhesion of malignant T cellular tumor to epithelial cells, making it easy to metastasize.

Both E-cadherin and N-cadherin belong to classical cadherins. Although they mediate cell-cell adhesion through the same mechanism, their actual effects are completely contradictory. Loss of E-cadherin is usually associated with the appearance of N-cadherin in a phenomenon referred as “the cadherin switch.” Researches suggest that E-cadherin could switch to N-cadherin to result in an increase of N-cadherin and a decrease of E-cadherin. Thus, cancer cells are transformed from a benign phenotype to an malignant phenotype.\(^3,13,14\) In vitro studies also reveal that the invasive capacity of N-cadherin negative cancer cells is significantly increased after transfection with N-cadherin. In the mean time, cell adhesion mediated by E-cadherin is depleted, which means that the expression of E-cadherin is decreased.\(^4\)

We found that with the increase of the infiltration depth, the decrease of the differentiation degree and the development of ESCC metastasis, the positive rate of E-cadherin is decreased, accompanied by an increase of N-cadherin, suggesting that a switch of E-cadherin to N-cadherin may occur in ESCC. Our findings are consistent with the report by Rosivatz, et al.\(^15\) As a result, E-cadherin and N-cadherin could be used as indices to predict the infiltration and metastasis of ESCC. Combined detection of the two indices may screen out patients with high metastatic potentials, thereby help them to obtain timely treatments, or be exempt from unnecessary adjuvant chemotherapy.

Silencing of N-cadherin gene in EC9706 using retrovirus mediated RNAi. In order to further validate the relationship of N-cadherin gene expression with infiltration and metastasis of ESCC, retrovirus mediated RNA interference technology was used.
to inhibit the gene expression of N-cadherin in EC9706 cells. We did not detect any significant changes in E-cadherin mRNA or protein expressions of stably interfered EC9706 cells, while both mRNA and protein expressions of N-cadherin and MMP-9 were downregulated. Additionally, the invasive capacity of EC9706 cells in vitro was decreased, along with the decrease of mRNA and protein expressions of N-cadherin and MMP-9.

Infiltration and metastasis of tumor cells involve the detachment of tumor cells from the primary foci via adhesive attraction, migration to the basal membrane, degradation of the extracellular matrix, breakthrough of the tissue barrier, local infiltration, and long distance and systematic diffusion. After introduction of exogenous N-cadherin gene into E-cadherin positive breast cancer cells, Nieman et al.\(^6\) find that, though cells are still positive for E-cadherin, their migration capability and invasiveness are increased. We also observed a significant decrease of EC9706 cell invasion after silencing N-cadherin gene, although there were no significant changes in E-cadherin mRNA or protein expressions in vitro. This implies that N-cadherin is more critical than E-cadherin in inducing tumor infiltration and metastasis.

Suyama et al.\(^5\) show that increased N-cadherin could induce gene expression of matrix metalloproteinases (MMPs) via FGF mediated activation of the MAPK-ERK signaling pathway, which could facilitate the formation of tumor vessels, and then growth and metastasis of tumors. MMP-9 is a protease family containing Zn\(^{2+}\) and Ca\(^{2+}\). Its main substrates are type IV/V collagen, therefore it is also called type IV collagenase (gelatinase). Type IV collagen is a structural protein in the basal membrane, which is very important to maintain the integrity of basal membranes. Because of the distinct helical structure of type IV collagen, it is resistant to most proteases. However, MMP-9 can degrade type IV collagen and laminin to damage the integrity of the basal membrane,\(^17\)\(^-\)\(^20\) which is the prerequisite of tumor infiltration and metastasis. We detected MMP-9 mRNA and protein expressions in EC9706 cells before and after the transfection of N-cadherin RNAi, and found that the mRNA and protein expressions of MMP-9 were decreased with the decrease of N-cadherin. The reduced N-cadherin may lead to the degradation of the extracellular matrix, and then decrease the invasive capacity of EC9706 via down-regulation of MMP-9. However, how does N-cadherin exert its effect via MMP-9 needs further investigation.

In summary, down-regulation of E-cadherin and up-regulation of N-cadherin may be involved in the genesis of ESCC. Silencing N-cadherin using RNA interference could inhibit the invasiveness of EC9706 cells in vitro. Our study may provide a new insight for investigating the invasive capacity of ESCC and exploring a new target for its clinical treatment.

Acknowledgements

Grant: National Natural Science Foundation of China (No. 072102310054)

References