ARTICLES

Relationship Between the Expression of NEDD9 in Gastric Cancer and the Invasion and Metastasis of the Tumor

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OBJECTIVE: To clarify the relationship between the expression of NEDD9 in gastric cancer and the invasion and metastasis of the tumor.
STUDY DESIGN: The postoperative pathological samples of 40 patients with gastric cancer receiving surgical resection from January 2014 to August 2016 in our hospital were selected as subjects. All gastric cancer tissues were confirmed as gastric adenocarcinoma by histopathological examination, and the patients did not receive any treatment before surgery. Meanwhile, paracancerous tissues <3 cm away from the foci were collected. HE staining showed that 24 cases had moderate-severe atypical hyperplasia; of those, 20 were taken for experimental studies. In addition, 40 cases of normal gastric mucosal tissues confirmed by gastroscopy and pathological examination were selected as controls. All samples were subjected to immunohistochemical assay, in situ hybridization, RT-PCR, and Western blot.
RESULTS: The positive expression rates of NEDD9 protein in normal gastric mucosal tissue, paracancerous atypical hyperplastic tissue, and gastric cancer tissue were 7.5%, 20%, and 92.5%, respectively. The NEDD9 mRNA expressions in normal gastric mucosal tissue, paracancerous atypical hyperplastic tissue, and gastric cancer tissue also followed an ascending order.
CONCLUSION: The overexpression of NEDD9 was closely related to the onset and progression of gastric cancer, and increase in its expression may be an early warning event. (Anal Quant Cytopathol Histpathol 2017;39:247–253)

Keywords: gastric cancer, invasion, metastasis, NEDD9.

Gastric cancer is one of the most common gastrointestinal malignancies, with the characteristics of high incidence, occult onset, easy metastasis, and high mortality.[1] At present, the incidence and mortality rates of gastric cancer rank second and third, respectively, among all malignant tumors. Therefore, it is necessary to further investigate the pathogenesis of gastric cancer and to search for more effective therapies.

NEDD9 is an important cytoskeletal protein.[2] The regulation on its expression may affect many biological processes, such as cell cycle, apoptosis, and migration. NEDD9 plays different roles in different tumor cells.[3] It is highly expressed in invasive and metastatic tumor tissues but lowly expressed in cells with weak tumorigenic ability. In this study, the immunohistochemical SP method and *in situ* hybridization were used to detect the protein and mRNA expressions of NEDD9 in 40 cases of gastric carcinoma tissue, 20 cases of

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paracancerous atypical hyperplastic tissue, and 40 cases of normal gastric mucosal tissue, aiming to explore its relationship with tumor invasion and metastasis.

Materials and Methods

Materials

The postoperative pathological samples of 40 patients with gastric cancer receiving surgical resection from January 2014 to August 2016 in our hospital were selected as subjects (Table I). The patients included 27 males and 13 females aged between 32 and 71 years old, with an average age of 55. All the gastric cancer tissues were confirmed as gastric adenocarcinoma by histopathological examination, and the patients did not receive any treatment before surgery. Meanwhile, samples were taken from paracancerous tissues <3 cm away from the foci. Through HE staining, 24 cases were found to be moderate-severe atypical hyperplasia; of those, 20 were taken for experimental studies. Forty cases of normal gastric mucosal tissue confirmed by gastroscopy and pathological examination were selected as controls. All samples were embedded in paraffin and sectioned for immunohistochemical assay and in situ hybridization.

Immunohistochemical Assay

Section Preparation. Paraffin sections were sliced continuously at a thickness of 4 μm in triplicate, with 1 conducted with NEDD9 immunohistochemical staining, 1 with PBS instead of primary antibody as negative control, and 1 as back-up.

Immunohistochemical Staining. SP immunohistochemical staining kit was purchased from Zymed Laboratories (USA). Mouse anti-human NEDD9 monoclonal antibody and rabbit anti-human NEDD9 polyclonal antibody were purchased from Santa Cruz Biotechnology (USA). The staining processes were performed in accordance with the kit's instructions.

Results Determination. The staining results were determined using unified scoring criteria and double-blinded method for determination and scoring by 2 pathologists totally unaware of any clinical and pathological data. The experimental results were determined and scored, and the positive expression of NEDD9 protein was determined by the level 2 scoring method. Scores for percentage of number of positive cells to the total number were as follows: 0 for positive cells <1%, 1 for 2~25%, 2 for 26~50%, 3 for 51~75%, and 4 for >75%. Scores for staining intensity were as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. Both the scores of a sample were multiplied as the total score. The samples were then classified according to the following scores: – (0~1 point), + (2~4 points), ++ (5~8 points), and +++ (9~12 points).

In Situ Hybridization

Paraffin sections were deparaffinized and hydrated with ethanol solutions at series of concentrations until distilled water. Endogenous catalase was inactivated and permeabilized to expose mRNA nucleic acid fragment, which was then fixed, prehybridized, hybridized, washed, and dropwise added freshly prepared BCIP/NBT substrate solution for color development in dark, then dehydrated by gradient concentrations of ethanol solutions, transparentized by dimethyl benzene, and mounted with neutral resin.

Design of Control Group. Sections were incubated in hybridization solution without labeled probe as negative control during hybridization steps.

Results Determination. Ten visual fields were selected at high magnification and determined according to staining intensity and percentage of

Table I *Baseline Clinical Data of Patients*

Clinical data	Case no.
Age (years)	
<60	17
≥60	23
Gender	
Male	29
Female	11
Tumor size (cm)	
<5	25
≥5	15
Invasion depth	
$T_{1\sim2}$	18
$T_{3\sim4}$	22
Differentiation grade	
I–II	26
III	14
No. of metastasized lymph nodes	
<3	17
≥3	23

positive cells: 0 for positive cells <1%, 1 for 2~25%, 2 for 26~50%, 3 for 51~75%, and 4 for >75%. Score for staining intensity: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. Both scores of a sample were multiplied as the total score. The samples were then classified according to the scores: – (0–1 point), + (2~4 points), ++ (5~8 points), and +++ (9~12 points).

Statistical Analysis

All data were analyzed by SPSS 13.0 and expressed as mean±standard variation ($\bar{x}\pm s$). The samples were compared by the χ^2 test. The categorical data were detected by the *t* test or analysis of variance. $P<0.05$ was considered statistically significant.

Results

Expression of NEDD9 in Tissues

Immunohistochemical Examination. NEDD9 protein expression in gastric cancer tissue, peritumoral atypical hyperplastic tissues, and normal gastric mucosal tissue were detected by SP immunohistochemistry. The results showed that NEDD9 existed mainly in the cytoplasm, stained brownish yellow, with relatively high expression in gastric cancer tissue (Figure 1). After statistical analysis, the positive rate of NEDD9 expression in gastric cancer tissue was 92.5% (37/40), 40.0% (8/20) in peritumoral atypical hyperplastic tissue, and 7.5% (3/40) in normal gastric mucosal tissue, among which the difference was statistically significant ($p<0.01$) (Table II). The score of immunohistochemistry of gastric cancer tissue was 5.88±0.46, higher than those of peritumoral atypical hyperplastic tissue (2.35±0.59) and normal gastric mucosal tissue (0.73±0.15) (Figure 2), in which χ^2 value was 58.534, $p<0.01$. There was significant difference among the groups, indicating that NEDD9 protein showed significantly high expression in gastric cancer tissue and low expression in normal gastric mucosal tissue.

Relationship Between the NEDD9 Expression in Gastric Cancer Tissue and Clinical Characteristics. Statistical analysis was conducted through analysis of the experimental results of immunohistochemistry in 40 cases of gastric cancer samples, combined with the patients' clinical data (gender, age, tumor size, tumor differentiation level, degree of infiltration, and lymph node metastasis number). The results showed that NEDD9 protein expression was not related to gender, age, tumor size, or differentiation level of the patients ($p>0.05$) but was positively correlated with the degree of infiltration and lymph node metastasis ($p<0.05$) (Table III).

Expression of NEDD9 mRNA in Tissues

In Situ Hybridization Detection. Through probe design, the in situ hybridization technique was adopted to detect the NEDD9 mRNA expression in tissue of each group. The results showed that NEDD9 mRNA existed mainly in the cytoplasm, with the positive signal bluish purple, and its expression levels successively decreased in gastric cancer tissue, peritumoral atypical hyperplasia tissue, and normal gastric mucosal tissue. The score of NEDD9 mRNA expression in gastric cancer tissue was 5.325±0.47, higher than those of peritumoral atypical hyperplastic tissue (2.6±0.59) and normal gastric mucosal tissue (0.875±0.19), which showed significant difference by statistics among the groups ($\chi^2=36.202$, $p<0.01$) (Table IV) (Figure 3).

Relationship Between the NEDD9 mRNA Expression in Gastric Cancer Tissue and Clinical Characteristics. Statistical analysis was conducted through analysis of the experimental results of in situ hybridization in 40 cases of gastric cancer samples, combined with the patients' clinical data (gender, age, tumor size, tumor differentiation level, degree of infiltration, and lymph node metastasis number). The results showed that NEDD9 mRNA expression was not related to gender, age, tumor size, or differentiation

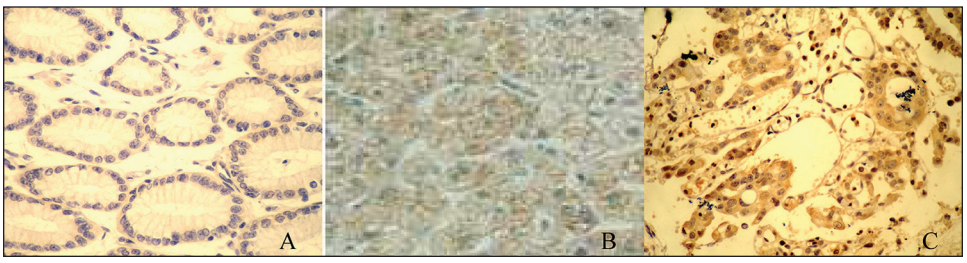

Figure 1
Expressions of NEDD9 in different tissues (SP, ×400). (A) Normal gastric mucosal tissue, (B) peritumoral atypical hyperplastic tissue, and (C) gastric cancer tissue.

Table II *Expression of NEDD9 in Tissues*

Group	–	+	++	+++	Positive rate (%)	χ^2 (p value)
Gastric cancer tissue	3	10	21	6	92.5	
Peritumoral atypical hyperplastic tissue	12	4	3	1	40.0	58.534 (<0.01)
Normal gastric mucosal tissue	37	2	1	0	7.5	

level of the patients ($p>0.05$) but was positively correlated with the degree of infiltration and lymph node metastasis ($p<0.05$) (Table V).

Discussion

Gastric cancer is one of the most common gastrointestinal malignancies, with the characteristics of high incidence, occult onset, easy metastasis, and high mortality. With the popularization of gastroscopy, laparoscope, barium meal, and other inspection methods, the detection rate of gastric cancer has improved to some extent. Currently, major therapeutic methods of gastric cancer include surgery, radiotherapy, chemotherapy, and other comprehensive treatments. For patients with advanced gastric cancer, complete cure is difficult with surgery, and chemotherapy has great toxicity and side effects and adverse reactions; meanwhile, there are problems such as tumor multidrug resistance and immune system damage and other issues, so the therapeutic effect is not satisfactory. In recent years, with continual advancement of research on the pathogenesis of tumors and further understanding of oncogenes and cancer suppressor genes, the treatment of gastric cancer has developed from comprehensive therapy based on surgical treatment into new treatments such as biological therapy, gene therapy, and immunotherapy. It is of positive significance to the prevention and treatment of gastric cancer by identifying genes related to the occurrence and development of gastric cancer and screening gene targets in early diagnosis through the research on genes related to gastric cancer.

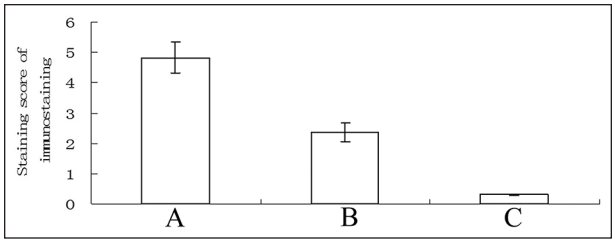

Figure 2 Immunohistochemical examination score. (A) Gastric cancer tissue, (B) peritumoral atypical hyperplastic tissue, and (C) normal gastric mucosal tissue.

NEDD9, also called HEF1 (human enhancer of filamentation 1) or Cas-L (crk-associated substrate-related protein, lymphocyte type), is a gene found in 1992 that is expressed in the embryonic brain of mouse but not expressed in the brain of the mature mouse.[4] As a member of adaptor molecule CAS (crk-associated substrate) group, NEDD9 was initially considered to have the same effects as other CAS group proteins—that is, it plays a role only in cell adhesion and migration; however, further studies found that NEDD9 has a more complicated regulatory mechanism and biological effects. As a skeleton protein, human NEDD9 has not been found to have enzymatic activity so far; however, it plays an important role in the molecular signaling pathway related to tumor metastasis and it can have the function of hub by connecting the upstream input signal with downstream effector molecules. Through NEDD9 protein expression and regulation, many biological activities can be regulated and controlled, such as cell growth, migration, adhesion, apoptosis, and signal transduction.[5]

NEDD9 expression and regulation is a series of complex and dynamic processes, through which many different biological processes can be influenced. NEDD9 regulation includes phosphorylation, transcriptional activation, and proteolysis process of NEDD9. In resting cells, NEDD9 has a very low expression level; however, when cells are induced to enter the cell cycle, NEDD9 expression can rapidly increase.[6] Though further in-depth understanding of NEDD9 expression and regulation is required, some inducible factors have been found to have a regulation function in NEDD9 expression. For example, TGF-β can induce transcription of NEDD9 mRNA, thus causing protein ex-

Table III *Relationship Between the NEDD9 Expression in Gastric Cancer Tissue and Clinical Characteristics*

Clinical factor	N	Immunochemical score	*F(t)*	p Value
Age (years)			0.394	0.731
<60	17	5.75±0.11		
≥60	23	5.77±0.08		
Gender			0.636	0.533
Male	29	5.77±0.10		
Female	11	5.74±0.11		
Tumor size			1.245	0.229
<5	25	5.74±0.12		
≥5	15	5.79±0.04		
Invasion depth			3.752	<0.001
T_{1-2}	18	4.694±0.10		
T_{3-4}	22	6.816±0.03		
Differentiation grade			0.686	0.501
I–II	26	5.80±0.10		
III	14	5.81±0.12		
No. of metastasized lymph nodes			3.498	0.03
<3	17	4.687±0.11		
≥3	23	6.808±0.04		

pression.[7] In 2 different retinoblastoma cell lines, a metabolite of vitamin A, atRA, can induce NEDD9 transcriptional expression, indicating that NEDD9 is related to nerve cell development.[8] In the rat model with brain ischemia, NEDD9 has a high expression in neuronal cells in the cerebral cortex and hippocampus.[9] Research on ovarian cancer and melanoma cells have found that stimulating NEDD9 expression is an important process in cancer metastasis.[10,11] This study found that the expression of the NEDD9 protein increased successively in normal gastric mucosal tissue, peritumoral atypical hyperplastic tissue, and gastric cancer tissue, with the positive expression rates of 7.5%, 20%, and 92.5%, respectively. The relative expression of protein detected by Western blot also increased successively. At the same time, the NEDD9 mRNA expression in normal gastric mucosal tissue, peritumoral atypical hyperplastic tissue, and gastric cancer tissue were detected by in situ hybridization and RT-PCR technique, and the results also showed a successive increase. This indicates that overexpression of NEDD9 is closely related to the occurrence and development of gastric cancer and its increase in expression might be an early event in the occurrence and developmental process of gastric cancer. This result is similar to that of overexpression of NEDD9 in other malignant invasive tumors.

Normal cell migration has a positive effect on the body and can promote embryonic development and inflammatory reaction; however, in tumor cells the function of cell migration is activated abnormally, resulting in invasive metastasis of tumor cells. NEDD9, located in the adhesion plaque of cells, exerts an influence on cell migration by adjusting the interaction of important molecules in cell migration. *In vitro* experimental studies on a few different cells have shown that NEDD9 overexpression can enhance the random migration speed and chemotaxis of cells,[12-14] while the reduction of NEDD9 expression can cause decreased

Table IV *Positive Rates of NEDD9 mRNA in Situ Hybridization Detection*

Group	–	+	++	+++	Positive rate (%)	χ^2
Gastric cancer tissue	4	14	17	5	90.0	36.202
Peritumoral atypical hyperplastic tissue	10	5	5	0	50.0	
Normal gastric mucosal tissue	35	3	2	0	12.5	

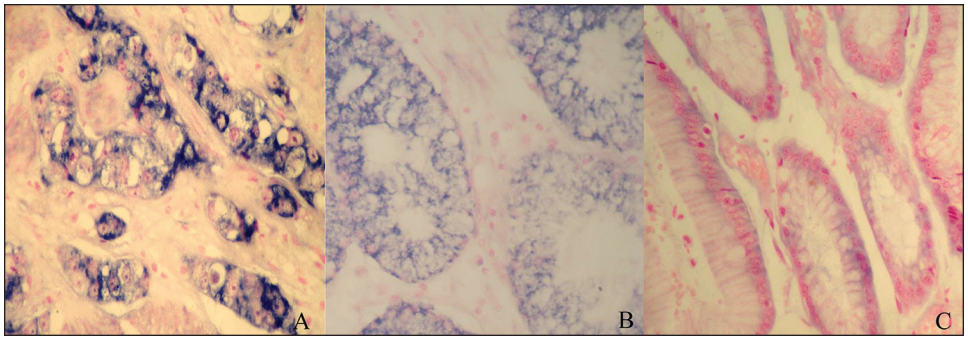

Figure 3
Expressions of NEDD9 mRNA in different tissues (ISH, BCIP/NBT, ×400). (A) Gastric cancer tissue, (B) peritumoral atypical hyperplastic tissue, and (C) normal gastric mucosal tissue.

chemotaxis of cells and cells with NEDD9 overexpression can stretch more easily[13]; moreover, C-terminal peptide with NEDD9 overexpression can cause cell rounding and loss of adhesion junction among cells.[15] Though there has not been comprehensive research on the migratory path caused by NEDD9, it has been found that NEDD9 can interact with some cell factors that can promote cell migration and invasiveness, causing its activation and expression. Research on melanoma,[16] breast cancer,[17] and glioblastoma[18] have found that NEDD9 overexpression plays a vital role in the migration process of tumor cells. This study found that the expression level of NEDD9 increased successively in normal gastric mucosal tissue, peritumoral atypical hyperplastic tissue, and gastric cancer tissue, and meanwhile, the positive expression of NEDD9 had no significant relationship with the age, gender, tumor size, or tumor differentiation grade of gastric cancer patients ($p>0.05$) but was related to the infiltration depth of tumor and lymphatic metastasis ($p<0.05$), indicating that NEDD9 might play a positive role in lymphatic metastasis and distant diffusion of gastric cancer, which is consistent with the research results of NEDD9 in other malignant invasive tumors.

The increase of NEDD9 expression can produce a variety of controls on cell growth. In normal cells or tumor cells at the early stage, the increase of NEDD9 expression can enhance cell migration and invasion capacity and meanwhile cause post-

Table V *Relationship Between the NEDD9 mRNA Expression in Gastric Cancer Tissue and Clinical Characteristics*

Clinical factor	N	NEDD9 mRNA expression	*F(t)*	p Value
Age (years)			1.278	0.211
<60	17	0.820±0.206		
≥60	23	0.894±0.125		
Gender			0.778	0.443
Male	29	0.826±0.200		
Female	11	0.877±0.153		
Tumor size			1.169	0.251
<5	25	0.821±0.215		
≥5	15	0.890±0.123		
Invasion depth			6.280	<0.001
T_{1-2}	18	0.647±0.143		
T_{3-4}	22	0.932±0.101		
Differentiation grade			1.148	0.260
I–II	26	0.838±0.171		
III	14	0.906±0.153		
No. of metastasized lymph nodes			5.342	<0.001
<3	17	0.691±0.158		
≥3	23	0.937±0.103		

mitotic defects related to the failure of cytoplasm movement. If it is cut into fragments, it might cause adhesion and cell apoptosis. However, NEDD9 might have different effects in the tumorigenesis of different types. Therefore, when different tumor cells have NEDD9 overexpression, we should have different conclusions. For example, in solid tumor and hematopoietic tissue tumor, the invasion of hemocyte to other tissues is a normal phenomenon. Therefore, if NEDD9 overexpression is detected, we cannot judge whether it is tumor invasion or not; however, for epithelial cells, NEDD9 overexpression can be identified as invasive solid tumor. Currently, NEDD9 overexpression is a conspicuous biomarker in metastatic melanoma[19] which can promote lung metastasis of the tumor and be used as a diagnostic basis in tumorigenesis and development. However, in breast cancer cell MDA-MB231, NEDD9 expression decreases, indicating that NEDD9 has different effects in tissue cells of different tumors. Research on the regulatory effect of NEDD9 expression in gastric cancer on cell growth of gastric cancer has just begun, and further in-depth studies on its effect on the development of gastric cancer are required.

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