

Study on effect of the expression of siRNA in gastric cancer bearing nude mice transplanted tumor NEDD9 gene

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Abstract: The clinical study found that NEDD9 showed high expression on the invasion in gastric cancer tissues and metastasis of the tumor. Based on promoting the fundamental role (Sisen *et al.*, 2013) to the expression level, the author further study NEDD9 siRNA, which could significantly reduce NEDD9 protein and mRNA in gastric cancer BGC823 cells, inhibition of cell proliferation, induce cell apoptosis, and decrease the invasiveness of gastric cancer cells, suggesting that NEDD9 plays an important role in the gastric cancer cell proliferation, apoptosis and invasion force. Through constructing a model transplanted gastric cancer in nude mice, the author observes the effect of NEDD9 siRNA on the growth of gastric cancer x-engrafts, and application of NEDD9 immunohistochemical SP method. The author also uses Western blot method to detect the gastric carcinoma in nude mice transplanted tumor tissues expression; applies situ hybridization, RT-PCR technology to detect the gastric cancer engraft tissues in NEDD9 mRNA. In order to further explore the relationship between NEDD9 and the development of gastric cancer, he provides a theoretical basis for the NEDD9 targeted therapy.

Keywords: NEDD9, gastric cancer.

INTRODUCTION

Gastric cancer is one of the most common malignant tumors. It takes the third place in the incidence of male malignant tumor and takes the fourth place in the incidence of female malignant tumor. East Asia included China is the area with the highest incidence of gastric cancer and mortality in the world (Wang and Morrow, 2000). The development of modern molecular biology make us realize that malignant tumor is a complex process with multi-step, multi-stage and progressive course and polygenes, multi-factor and polymolecule participated in. neural precursor cell expressed developmentally down-regulated 9 (NEDD9) is a gene related to invasion and metastasis and is one member of Crk-associated substrate (CAS) family. Like other members of CAS family, NEDD9 showed high expression when many tumor transfer. The expression amount of NEDD9 is different in different types of tissues and cells as well as different growth condition. In normal human tissues such as lung and gastric tissue, expression level of NEDD9 mRNA and protein is high in tissue that contains rich immature lymphocyte and the brain of fetus (although the expression decrease in adult period). Tumor cell lines, lymphoma cell lines, malignant glioma strains and melanoma cell lines which come from epithelial cell are all rich in NEDD9. However, there has no study on the expression of NEDD9 in gastric cancer cells. And the expression of NEDD9 and clinical pathological feature of gastric cancer need to be further studied.

Research found that NEDD9 was the stimulating factor before the metastasis of melanoma (Vogel *et al.*, 2010) and spongioblastoma, and the change of NEDD9 expression can affect the metastasis of breast cancer. Thus the literatures had mutual contradiction. Further study found that the expression of NEDD9 showed positive relationship with the loss of estrogen receptor in tumor specimens (Knutson *et al.*, 2008; Merrill *et al.*, 2004; Richer *et al.*, 2002). Research proved that NEDD9 showed high expression in hepatic metastatic tumor tissue sample and hepatic cancer cell strains with high metastasis (Buterin *et al.*, 2006). Li et al. studied and found that the expression of NEDD9 in colon cancer cell lines was higher than that in normal colon cancer cell lines (Monroe *et al.*, 2003). For the cell lines of gastrointestinal stromal tumor (GIST) that is drug resistant to imatinib, transcription of NEDD9 was highly up-regulated (Martin-Rendon *et al.*, 2007). Therefore, NEDD9 might perform different functions in different tumor and cell metastasis of gastric tumor.

So far, there has no research on the relationship between NEDD9 and gastric cancer at home and abroad. This paper adopted SP method and Western Blot method to detect the NEDD9 expression in nude mouse transplantation tumor tissue, applied hybridization *in situ* and RT-PCR technology to detection the expression of NEDD9 mRNA in transplantation tumor tissue of gastric cancer, and thus discuss the relationship of NEDD9 and the occurrence and development of gastric tumor in order to provide theoretical basis for targeted therapy of gastric cancer.

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MATERIALS AND METHODS

Experimental animal and cell line

15 nude mice with thymus defects BALB/C were purchased from the Chinese Academy of Sciences, Shanghai slyke experimental animal Co., Ltd., nude mice 4 weeks yearling, female, weight 18-22g; gastric carcinoma cell line BGC823, were purchased from the CAS Shanghai Institute of life science cell library.

Experimental methods

Animal grouping and cell preparation

The BALB/C mice were randomly divided into 3 groups, 5 rats in each group, which were divided into blank control group (injection of untransfected BGC823 cells), negative control group (injection of non-specific siRNA transfection of BGC823 cells), experiment group (injection of transected NEDD9 siRNA-2 cells; BGC823); by liposome transfection technique, specific and nonspecific siRNA were transfected into gastric carcinoma cell line BGC823, and collected non transfected gastric cancer BGC823 cells which can be adjusted cell density to $1 \times 10^7/\text{ml}$.

Establishment of transplanted tumor model of human gastric cancer in nude mice

Iodophor disinfection (vaccination was sited into the right forelimb armpit skin), syringe aspiration cell suspension 0.2ml (concentration $1 \times 10^7/\text{ml}$) were injected into the subcutaneous inoculation, sterile cotton swab gently press the point of puncture 2min and iodophor disinfection; The experimenter paid attention to activities, eating and mental state through daily observations in nude mice after inoculation. Visible to the naked eye the tumor mass after every 3 days, the experimenter used Vernier caliper to measure tumor size (L) and short diameter (S). The experimenter used the formula $V (\text{mm}^3) \text{ calculation of tumor volume} = L * S^2 * 0.5$ (L.R., 2004). Based on Tumor Tissue Specimens, nude mice in inoculation of tumor cells after 5 weeks were executed quickly, stripped the masses, and measured the tumor volume. The tumor was composed of two parts, one part was preserved in liquid nitrogen rapidly and the other part with 40g/L para formaldehyde fixed paraffin embedded sections to make for section to be reserved.

Expression of NEDD9 protein by immunohistochemistry to detect the tumor tissues

4 μm of paraffin sections serial sectioned three copies: one copy was stained by NEDD9 immunohistochemical, one use of PBS instead of the first antibody as negative control, the other as a spare slice. SP immunohistochemistry staining kit was purchased from ZYMED Company USA, mouse anti human NEDD9 monoclonal antibody and Rabbit anti human NEDD9 polyclonal

antibody were purchased from Cruz Company USA Santa, dyeing process of SP series kit according to instructions.

Using the unified standard and double blind method to judge the staining results, the experimental results were judged, scored by two pathologists and two scoring methods (Li *et al.*, 2004) to determine the expression of NEDD9 protein sample. The percentage of total number of cells scoring positive cell numbers: <1% positive cells was 0, from 2 to 25% was 1, from 26 to 50% was 2, from 51 to 75% was 3, >75% was 4. Staining intensity score: no staining was 0; weak staining for 1; moderate staining for 2 points; strong staining for 3 points. The samples both were recorded as total score to be multiplied, in accordance with the definition of fractional sample into four categories: 0~1 (-), 2~4(+), 5~8(++), 9~12(+++).

Expression of NEDD9 protein was detected by blot western in transplanted tumor tissue

Extraction of total tissue protein: fetched fresh tissue, cutting, grinded into homogenate, packed and stored supernatant at -80°C ; prepared electrophoresis gel, electrophoresis; transferred film and antibody incubation: the gel of proteins transferred to nitrocellulose membrane and using TBS cellulose nitrate membrane soaked to the containing a closed liquid dish, room temperature closed 1h in decoloration table, then be closed for good film into hybrid bag, added 1ml diluted as 1:1000 (dilution as containing 5% skim milk powder (TBS-T) of an anti mouse anti human NEDD9 monoclonal antibody), sealing with 0.1% TPS-T membrane washing 3 second, every time 10min. Second antibody was (HRP labeled Goat anti mouse IgG) diluted 1000 times with 5%skim milk powder TBS-T. Room temperature with table 1H was incubated with TBS-T transfer film, washed 3 times, each time 5min. According to the specification of ECL Advance TM kit, the experimenter mixed a solution with B solution, gently dried with filter paper liquid transfer film on the preservation of the membrane. The protein side faced up, and ECL fluid in the membrane, got out 5min after the reaction, absorbed redundant ECL liquid with filter paper to wrap film with a clean plastic wrap. Developing and fixing in a dark room, X-ray film was cut into appropriate size, and put on the plastic wrap, then exposed 3min, put the membrane into the developer. The experimenter washed into the fixing solution for fixing until obvious bands stop developing. β -actin was tested in the same way as the test.

Expression of in situ hybridization in transplanted tumor of NEDD9 mRNA

The slice was dewaxed to water in conventional methods, inactivation of endogenous catalase, penetrated membrane, exposed mRNA nucleic acid fragment, fixed, prehybridization, hybridization, washed after hybridization and dropped BCIP/NBT substrate solution which was

freshly prepared. After nuclear fast red counterstaining, the dehydrated gradient alcohol, cleared xylene and sealed with neutral gum. Hybridization solution without labeled probe was regarded as a negative control activities section.

At higher magnification, 10 views were selected. According to the shade of color and the percentage of positive cells were seen as a judge, <1% positive cells was 0, 2 and 25% recorded as 1, 26~50% recorded as 2 points, from 51 to 75% for 3 points, >75% recorded as 4 points. Staining intensity score was as followed: no staining was 0; weak staining for 1; moderate staining for 2 points; strong staining for 3. The two samples were multiplied to record as the total score. According to the score, samples were divided into four categories: 0~1(-), 2~4(+), 5~8(++), 9~12(+++).

Expression of RT-PCR detected in transplanted tumor of *nedd9* mRNA

NEDD9 and β -actin primers were designed and synthesized by Shanghai Sangon Company, which extracted total RNA in the organization.

According to Trizol Kit (Invitrogen Company in America), extraction of total RNA in tumor tissue stored at -70° frozen; Determining RNA purity and integrity, UV spectrophotometer was detected A260/A280 value to get the purity and concentration of RNA; Taking 2μ l total RNA as the sample, he decided whether electrophoresis showed obviously the band 5S, 18S and 28S with 1% agarose gel, such as extracted RNA qualified; If that was yes, RT-PCR reaction included reverse transcribed to cDNA and PCR. According to analysis of RT-PCR Kit (Dalian TaKaRa Company), RT-PCR and PCR product generated the DNA bands of PCR products. Analysis system was used to scan analysis of DNA with Gel-Doc2000 gel imaging. According to the ratio of the density of NEDD9 and β -actin density, the author got the expression level of the NEDD9 mRNA.

STATISTICAL ANALYSIS

The author applied SPSS13.0 software for statistical analysis, using x2 test and t test. $P < 0.05$ had significant difference in statistics.

RESULTS

Establishment of transplantable model of gastric carcinoma in nude mice

Three groups of cells were injected subcutaneously in nude mice, which began to observe the formation of gastric cancer in 8~11 days. Xenograft tissue boundaries became clear; after transplantation tumor 5 weeks block, these became the form in a peanut size irregular shape with clear boundaries surrounding tissue. The mice were sacrificed, measured the tumor

volume, tumor volume was detected in blank control group (mm^3) which was 1912.83 ± 144.91 ; tumor volume in negative control group was 1812 ± 124.93 ; tumor volume in experimental group was 897.83 ± 99.23 ; there were significant differences in siRNA transfected with tumor volume compared with the other two control groups ($P < 0.001$). The results were seen in tables 1, 2, 3.

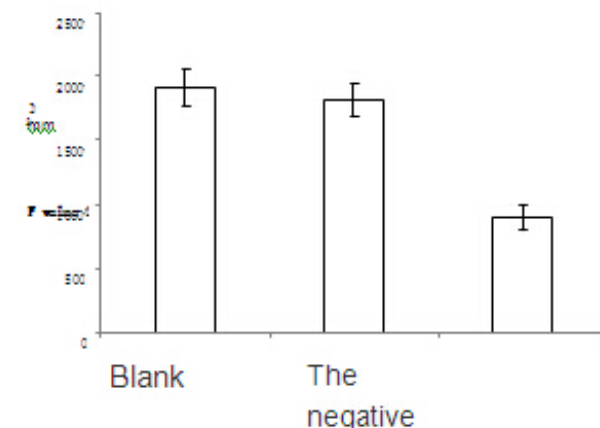


Fig. 1: Comparison of tumor volume of transplanted tumor of human gastric cancer in nude mice

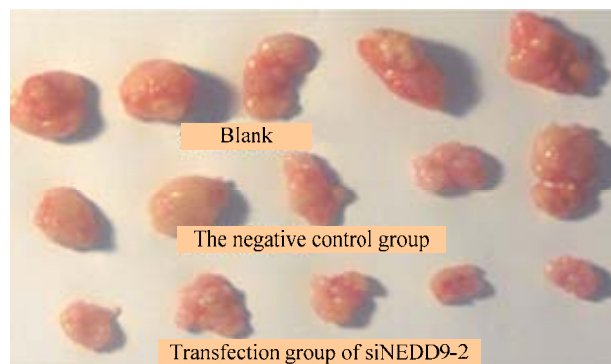


Fig. 2: Groups in transplanted tumor of gastric carcinoma in nude mice.

A blank control group; B. negative control group; C. NEDD9 siRNA transfection group.

Growth curves of groups of gastric cancer xenografts in nude mice

The cells were injected into nude mice, measured the tumor volume respectively at 1, 2, 3, 4, 5 weeks, then drawn the growth curve. The results showed that the tumor RNA interference group grown slowly, and the volume was smaller than the other two groups ($P < 0.05$), as seen in fig. 4.

Expression of NEDD9 protein in groups of gastric carcinoma xenografts in nude mice

Immunohistochemical Examination Results

Compared with blank control group and negative control group, the expression of NEDD9 protein in NEDD9 transfected with siRNA tumor tissue was significantly

decreased and the difference was statistically significant ($P < 0.05$) as seen in table 2, fig. 5.

Test results of western blot

The relative expression of NEDD9 protein of Western blot was detected in gastric carcinoma xenografts in nude mice. The blank control group and negative control group were 0.843 ± 0.083 , 0.786 ± 0.075 , NEDD9 siRNA transfected group was 0.559 ± 0.083 . Compared with the control group, transplanted tumor tissue of NEDD9 protein decreased obviously. There was statistically significant difference ($P < 0.05$) as seen in table 3, fig. 6.



Fig. 3: Comparison of tumor volume in groups of gastric cancer xenografts

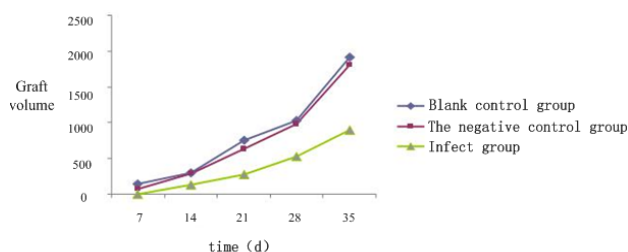


Fig. 4: The tumor body groups of gastric cancer xenograft growth in nude mice contrast curve

A. blank control group; B negative control group; C. NEDD9 siRNA transfection group

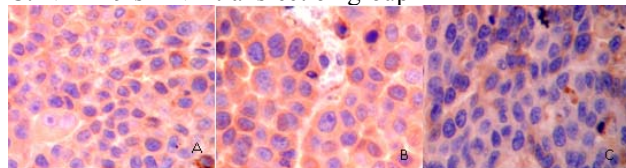


Fig. 5: Comparison of expression of NEDD9 protein in groups of gastric cancer xenografts in nude mice tissues (SP, *400)

Expression of gastric carcinoma xenografts in nude mice in NEDD9 mRNA

In situ hybridization results

Compared with the control group, the expression of NEDD9 siRNA transfection group transplanted tissues in

NEDD9 mRNA were weakened and the difference was statistically significant ($P < 0.05$) as seen in table 4, fig. 7.

A blank control group; B negative control group; C. NEDD9 siRNA transfection group

Test Results of RT-PCR technology

Relative expression of NEDD9 RT-PCR was detected in gastric carcinoma xenografts in nude mice mRNA. Blank control group and negative control group were 0.723 ± 0.058 , 0.664 ± 0.092 . NEDD9 siRNA transfected group was 0.497 ± 0.075 , compared with the two control groups. The expression of NEDD9 siRNA was transfected into tumor tissue in NEDD9 group mRNA, which decreased obviously, and the difference was statistically significant ($P < 0.05$). See table 5, fig. 8.

1. Blank control group; 2. negative control group; 3. siRNA transfection group; M. Mark

CONCLUSION

Gastric cancer (gastric, cancer, GC) is one of the most common malignant tumor of digestive tract. Its incidence ranks the fourth in malignant tumor, and the mortality rate ranks second only to lung cancer (Parkin *et al.*, 2001). At present, gastric cancer therapy includes operation, radiotherapy, chemotherapy, advanced gastric cancer, but the treatment effect is not ideal.

The occurrence and development of gastric cancer is a complex process of many proteins, oncogene, tumor suppressor gene and other factors involved in this paper (Chuanli and Wei, 2005). With the development of molecular biology, tumor-targeting therapy has become the focus of current research, which has made a big progress.

BALB/C mutant mice nude mice is a congenital defect due to the thymus, immune dysfunction both exogenous tumor cells without rejection, so it is widely used in the research of tumor animal model (Xuerui, 2007). The use of tumor animal model can occur on the development and changes in tumor research and intervention effect on tumor assessment. It is clinical research, which provides important reference materials (Alajez *et al.*, 2008; Abuzeid *et al.*, 2009). In this study, the gastric cancer BGC823 cells, siRNA interference after gastric cancer cell BGC823, negative control disturbance of gastric cancer BGC823 cells are injected into nude mice axillaries subcutaneous tissue that can form a visible tumor, which suggest that the transplanted tumor model of human gastric cancer in nude mice. After 5 weeks on the volume and three of the total group the tumor mass was measured, tumor volume siRNA interference group was significantly less than the blank control group and negative control group and the grown slowly that suggests gastric cancer cell in nude mice declined after NEDD9 gene silencing.

Table 1: Effect of NEDD9 on gastric carcinoma in nude micetransplanted tumor volume ($\bar{x}\pm s$ n=5)

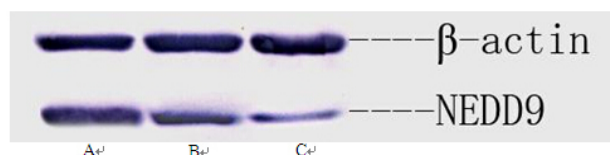
Group	Tumor volume (mm ³)	F value	P value
Blank control group	1912.83±144.91		
The negative control group	1812.00±124.93	121.168	<0.001
The experimental group (siRNA transfection group)	897.83±99.23		

Table 2: Effect of NEDD9 siRNA on NEDD9 protein ingastric carcinoma xenografts in nude mice. ($\bar{x}\pm s$ n=5)

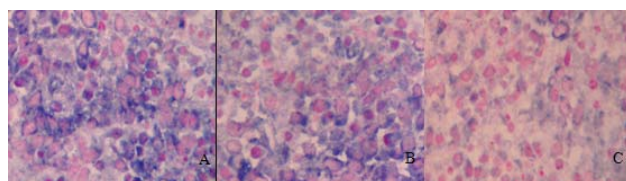
Group	The expressive of NEDD9 protein	F value	P value
Blank control group	9.28±1.04		
The negative control group	8.47±0.63	16.059	0.004
siRNA transfection group	6.04±0.35		

Table 3: Expression of NEDD9 protein in each nude mice transplantation tumor of stomach cancer tissues ($\bar{x}\pm s$ n=5)

Group	The expressive of NEDD9 protein	F	P
Blank control group	0.843±0.083		
The negative control group	0.786±0.075	20.871	<0.001
siRNA transfection group	0.559±0.083		

**Fig. 6:** Comparison of expression of NEDD9 protein in groups of gastric carcinoma xenografts in nude mice (SP, ×400).

Using immunohistochemistry, Western Blot and in situ hybridization, RT-PCR method was applied to detect the expression level of NEDD9 protein and mRNA in gastric carcinoma xenografts in nude mice in each group. It was found that siRNA interference group of NEDD9 protein and mRNA compared with blank control group and negative control group ($P<0.05$), which showed that siRNA can effectively reduce the expression level of NEDD9 in gastric cancer tissue.

**Fig. 7:** Comparison of the expression of gastric carcinoma xenografts in nude mice in NEDD9 mRNA (ISH, BCIP/NBT, ×400)

Since the discovery explored in the field of gene therapy, RNA interference technology has been widely studied. Chemotherapy killed tumor cells at the same time, for not targeting tumor cells, thus caused many normal cell death. Therefore it has a better specificity. Many studies show that RNA interference to many related gene in gastric cancer can achieve the purpose of inhibiting the growth of gastric cancer cells. But this

technology to clinical applications are still many problems need to be solved, whether can have the same effect in the human body? In the body of siRNA exogenous, will it cause unexpected harm to human body? Does it have different effect expression? It is a very human research data about the lack of siRNA when a treatment is not mature. With the further understanding of the mechanisms of RNA interference, RNA interference technology has further improved. The method will become a new direction of cancer gene therapy, which has a wide application prospect (Kim and Rossi, 2007).

NEDD9 as a structural protein, whose expression changes on the expression of downstream protein, has the function of NEDD9 in gastric cancer tissue. Why would the high expression of NEDD9 inhibit the expression of siRNA in gastric cancer after use? Signal factor leads to cancer cell biological activity change. All these problems need our further study in the future.

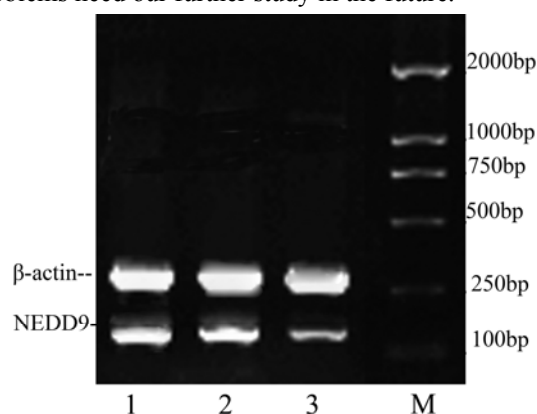
**Fig. 8:** Comparison of expression of RT-PCR was detected in gastric carcinoma xenografts in nude mice of NEDD9 mRNA

Table 4: Effect of siRNA on the expression of NEDD9 NEDD9 mRNA gastric carcinoma xenografts in nude mice (n=5)

Group	The expressive of NEDD9 protein	F value	P value
Blank control group	8.26±1.03		
The negative control group	8.22±1.04	8.343	0.019
siRNA transfection group	5.24±1.05		

Table 5: Effect of siRNA on the expression of NEDD9 NEDD9 mRNA tumor tissues (n=5)

Group	The expressive of NEDD9 protein	F value	P value
Blank control group	0.723±0.058		
The negative control group	0.664±0.092	16.290	<0.001
siRNA transfection group	0.497±0.075		

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