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Expression of 2 transcripts of *NGX6* gene in colorectal cancer and the correlation with carcinoembryonic antigen

SU Zheng[1], WANG Xiaoyan[1], SHEN Shourong[1], WANG Li[2], LI Yu[1], LI Nan[1], LI Zeng[1]

(1. *Department of Gastroenterology, Third Xiangya Hospital, Central South University, Changsha* 410013; 2. *Cancer Research Institute, Xiangya Medical School, Center South University, Changsha* 410078, *China*)

Abstract: **Objective** To investigate the expression and function of *NGX6-S* (short transcript) and *NGX6-L* (long transcript) in colorectal cancer. **Methods** In situ hybridization was used to detect the expression of these 2 transcripts in colorectal cancer tissues and paired normal tissues, and analyze the correlation between *NGX6* and carcinoembryonic antigen (CEA). **Results** The expression of *NGX6-S* was higher than that of *NGX6-L* in the colorectal cancer tissues ($P = 0.008$). The expressions of *NGX6-S* and *NGX6-L* were not different among the 4 stages of colorectal cancer ($P > 0.05$). The expression of *NGX6-S* in the colorectal cancer tissues of Duke A, B, and C stages had no difference with that of the paired normal tissues ($P > 0.05$). Inversely, the expression of *NGX6-S* in the colorectal cancer tissues of Duke D stage was lower than that in the paired normal tissues ($P = 0.033$). *NGX6-L* expression was not different between the colorectal cancer tissues and the paired normal tissues in 4 stages ($P > 0.05$). The expression of *NGX6-S* and *NGX6-L* had no correlation with the serum concentration of CEA. **Conclusion** *NGX6-S* may play an important role in colorectal cancer, and the lowered expression of *NGX6-S* may contribute to the distant metastasis of colorectal cancer.

Key words: colorectal cancer; *NGX6* gene; transcript; carcinoembryonic antigen

NGX6 基因两转录本在不同分期结直肠癌中的表达及与癌胚抗原的关系

苏峥[1], 王晓艳[1], 沈守荣[1], 王理[2], 李予[1], 李楠[1], 李曾[1]

(中南大学 1. 湘雅三医院消化内科, 长沙 410013; 2. 湘雅医学院肿瘤研究所, 长沙 410078)

[摘要] **目的**:探讨 *NGX6* mRNA 两种转录本 *NGX6-S* 和 *NGX6-L* 在结直肠癌中的表达及功能。**方法**:采用原位杂交法检测 *NGX6-L* 和 *NGX6-S* 在不同分期结直肠癌癌组织及其配对正常组织中的表达特

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Biography SU Zheng, master, mainly engaged in the research of pathogenesis of colorectal cancer.

Corresponding author WANG Xiaoyan, E-mail: wxy20011@163.com

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征,并分析其与血清癌胚抗原(CEA)的相关性。**结果**:在结直肠癌组织中短转录本 *NGX6-S* 的表达明显高于长转录本 *NGX6-L*($P=0.008$)。*NGX6-S* 和 *NGX6-L* 在不同分期结直肠癌癌组织中的表达差异均无统计学意义($P>0.05$)。*NGX6-S* 在 Duke A,B 和 C 三期结直肠癌组织中的表达与其在配对正常组织的表达差异无统计学意义($P>0.05$),在有远处转移的 Duke D 期结直肠癌组织中的表达低于配对正常组织($P=0.033$)。*NGX6-L* 在 Duke A,B,C 和 D 期结直肠癌组织中的表达与配对正常组织差别无统计学意义($P>0.05$)。结直肠癌中 *NGX6-S*,*NGX6-L* 的表达与血清 CEA 浓度无相关性。**结论**:在结直肠癌中 *NGX6* 基因发挥抑瘤功能的主要形式是短转录本 *NGX6-S*,其表达下调可能与结直肠癌的远处转移相关。

[**关键词**] 结直肠癌; *NGX6* 基因; 转录本; 癌胚抗原

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NGX6 gene is a tumor-associated gene cloned in our lab, which is located at the region of chromosome 9p21-22, with the full length of cDNA 2.1kb, encoding protein with 2 transmembrane domains, an EGF-like domain and 3 N-glycosylation sites in the extracellular domain, and a tyrosine kinase sites in cytoplasmic region. The preliminary studies have shown that the expression of *NGX6* gene was downregulated in colon cancer tissues, especially obvious downregulation or deletion in the colon cancer with metastasis [1-2]. In vivo and in vitro proliferation and invasion tests have shown that *NGX6* can inhibit the proliferation and metastasis of colon cancer cells and the tumor angiogenesis [3-4]. Its tumor inhibition mechanism may be associated with *NGX6* negative regulation of Wnt /β-catenin pathway and EGFR-mediated JNK / SAPK pathway activity, downregulation of the downstream target genes cyclinE and cyclinD1 expression and delay the cell cycle progression of G_1 to S phase [5-6]. These results suggested that *NGX6* was a tumor suppressor gene candidate with great potential.

In recent years, our group discovered that *NGX6* mRNA could produce 3 different kinds of transcripts through different splicing ways, named *NGX6-1*, *NGX6-2*, and *NGX6-3*, respectively. *NGX6-1* is 3 747 bp, composed by 13 exons, and the 13 215-24 616 bp is the coding region; the *NGX6-2* is 3 474 bp, composed by 14 exons, encoding region is the same as that of *NGX6-1*. The 2 transcripts encode the same protein with 472 amino acids, called as long transcript *NGX6-L*. *NGX6-3* is 2 173 bp, composed of 11 exons, and the 13 215-17 969 bp is the coding region, encoding the protein of 338 amino acids, called short transcript *NGX6-S* (Fig. 1).

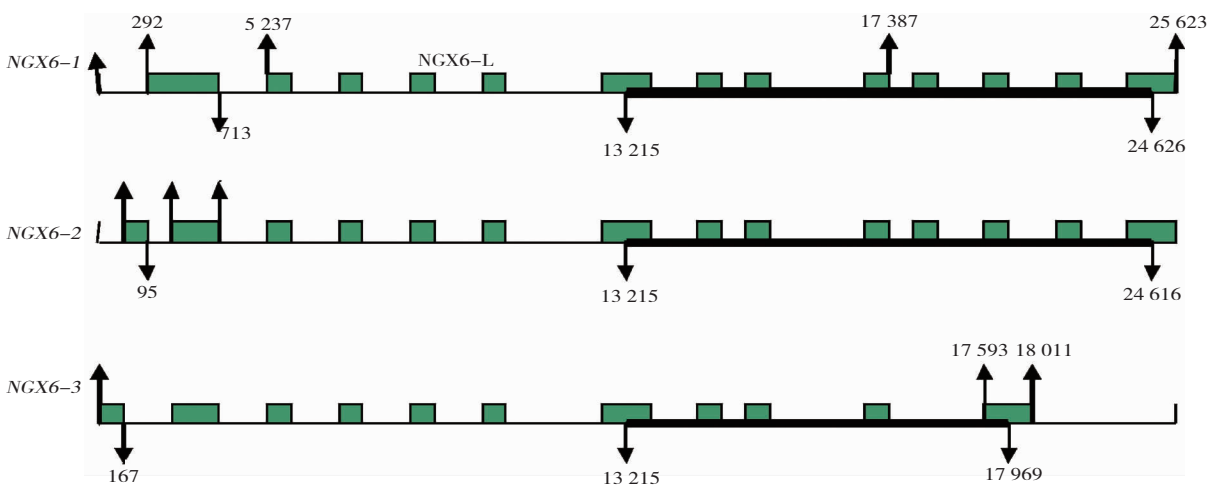

Fig. 1 Three transcripts of *NGX6* gene. *NGX6-1* and *NGX6-2* translate the same protein which contained 472 amino acids, which were named as *NGX6-L*. *NGX6-3* translates another protein containing 338 amino acids, and named as *NGX6-S*.

Gene transcriptional regulation is an important factor that affects its functions. One gene could produce different transcripts through different splicing ways. Different transcripts have different functions, so single gene functions present diversification. Then, what functions do the 2 transcripts of *NGX6* gene perform in the colorectal cancer? In this study, in situ hybridization was used to detect the expression of *NGX6-S* and *NGX6-L* in the colorectal cancer tissues and the paired normal tissues. By defining the characteristics of 2 *NGX6* transcripts in the colorectal cancer, and analyzing the relevance of expression with carcinoembryonic antigen (CEA), we provided experimental basis for function and mechanism of *NGX6* gene.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Tissue sections

One hundred cases of colorectal cancer paraffin blocks from June 2006 to March 2009 were obtained from Third Xiangya Hospital of Central South University, and staged according to the Dukes stage of colorectal cancer. They consisted of 12 Duke A stage, 42 Duke B stage, 22 Duke C stage, and 24 Duke D stage. For each case, the cancer tissues and paired normal colorectal tissues (normal tissue more than 5 cm away from caner tissue) were taken. The paraffin blocks were placed on the ice box cooling for 30 min before slicing, and kept under 4℃ for future use.

1.1.2 Design of oligonucleotide probes

The 819-1 017 bp of *NGX6-S* and the 819-1 419 bp of *NGX6-L* were specific sequences through bioinformatics prediction. The specific sequence regions of the both were selected to design and synthesize the oligonucleotide probes of *NGX6-S* and *NGX6-L*. The gene sequeces of mixed probes of *NGX6-S* were TTATCACTGGGTGCTTGTGCATGGTGGTGGGTCAC, TCCGAGGGTTTGGGAATGTCTGTGCCTTCACTGTG, and TGCATAGATAATGGTCATTTTTGTAAGACACATTG. and those of mixed probes of *NGX6-L* were ATCATGGACTACGATGTGCTGCGATTCTGTGATTT, CACTGCTACCCACCCACGTGGCGCCGCTGGCTTTT, and AACTACTTCTACATTCACAGCATTTGGCATATGCT.

1.1.3 Main reagents

NGX6-L and *NGX6-S* in situ hybridization detection kit (provided by Wuhan Boster Biological Engineering Co., Ltd.), including: pepsin (×10), pre-hybridization solution, *NGX6-L* and *NGX6-S* oligonucleotide probes hybridization solution, blocking solution, biotinylated mouse anti-digoxin, SABC-POD, and biotinylated peroxidase.

1.2 Methods

1.2.1 In situ hybridization

Tissue sections were deparaffinized, rehydrated, deproteinated, and fixed with paraformaldehyde. Then the sections were hybridized with pre-hybridization for 2 h at 42 ℃ and mixed probes of *NGX6* at 42 ℃ overnight. Following the SSC washing, and the egg white-milk and calf serum blocking, biotinylated mouse anti-digoxin and SABC were dripped subsequently. Then washed with PBC, dyed by DAB for 2-3 min, re-dyed with hematoxylin for 60 s, and sealed, the slides were observed under a microscope.

1.2.2 Criterion of staining degree

According to the positioning and staining degree of 2 transcripts of *NGX6* in the cytoplasm, the expression of gene in different Duke stages of colorectal cancer was identified. The determination standard referred to the literature [7]. (1) Intensity score was applied as follow: no reaction (score 0); light brown cells, weak intensity (score 1); cell staining into brown, moderate Intensity (score 2); cell presenting brown, strong immunostaining (score 3). (2) Score of the positive cells: the number of positive expression of cells < 5% (score 0);

the number of positive expression of cells was 5%-25% (score 1); the number of positive expression cells was 25%-50% (score 2); the number of positive expression of cells was more than 50% (score 3). The final score was the product of the scores obtained from the above 2 criteria. The results of pathological analysis were approved by 2 pathologists.

1.3 Statistical analysis

The statistical analysis was carried out with the statistical package SPSS 13.0. Paired t test was adopted for the paired measurement data, and 2-sample t test was adopted for the 2 independent group design, For multiple samples, analysis of variance was used; and for non-normal distribution measurement data, non-parametric test was adopted. For the enumeration data, chi-square test was adopted; and correlation test was adopted for the correlation analysis. The significant difference was determined by $P<0.05$.

2 RESULTS

2.1 Expression of *NGX6-S* and *NGX6-L* in colorectal cancer tissues and paired normal tissues

2.1.1 Differential expression of *NGX6-S* and *NGX6-L* in colorectal cancer tissues

The expression of 2 transcripts of *NGX6* was primarily localized in the cytoplasm area. The expression of *NGX6-S* in colorectal cancer tissues presented strong positive or positive, and the expression of *NGX6-L* in colorectal cancer tissues presented positive. The score of *NGX6-L* was 2.6300 ± 0.2658, and the score of *NGX6-S* was 3.3900 ± 0.3028, which suggested that the expression of *NGX6-S* was significantly higher than that of *NGX6-L* ($P=0.008$), and *NGX6-S* was the main expression form of *NGX6* gene in colorectal cancer tissues (Fig. 2).

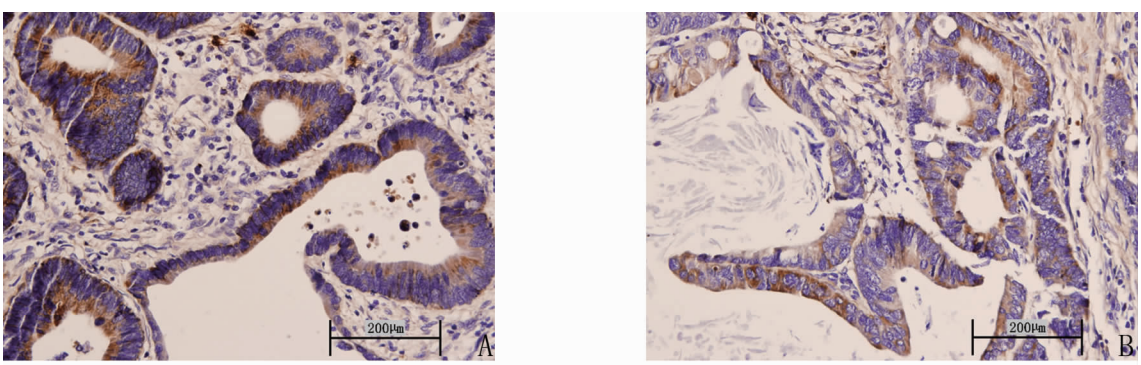

Fig. 2 Expressions of 2 transcripts of *NGX6* gene in the colorectal cancer by in situ hybridization. A: The strong positive expression of *NGX6-S* in colorectal cancer; B: The positive expression of *NGX6-L* in colorectal cancer.

2.1.2 Expression of *NGX6-S* and *NGX6-L* in different stages of colorectal cancer tissues and paired normal tissues

The expression of *NGX6-S* in colorectal cancer tissues of 4 stages was not different ($P=0.633$). The expression of *NGX6-S* in colorectal cancer tissues of the Duke A, B, and C stages has no difference with that of the paired normal tissues ($P>0.05$); while the expression of *NGX6-S* in colorectal cancer tissues of Duke D stage tissue was significantly down-regulated, compared with the paired normal tissues ($P=0.033$) (Tab. 1, Fig. 3).

The expression of *NGX6-L* in the colorectal cancer tissues of 4 stages was not different ($P=0.783$). The expression of *NGX6-L* between various stages of colorectal cancer tissues and paired normal tissues was also not different ($P>0.05$) (Tab. 1, Fig. 4).

Tab. 1 Expression of *NGX6-S* and *NGX6-L* in colorectal cancer and paired normal tissues of 4 stages colorectal cancer

Stages	*NGX6-S*			*NGX6-L*		
	Colorectal carcer	Normal tissues	*P*	Colorectal Carcer	Normal tissues	*P*
Dukes A ($n=12$)	4.0833 ± 0.7732	2.2500 ± 0.7295	0.140	3.1667 ± 0.8513	1.7500 ± 0.6643	0.192
Dukes B ($n=42$)	3.6829 ± 0.4907	2.7143 ± 0.4473	0.080	2.8049 ± 0.4179	2.6429 ± 0.3972	0.838
Dukes C ($n=22$)	3.1364 ± 0.6321	3.0909 ± 0.7084	0.948	2.5000 ± 0.5253	3.4545 ± 0.6955	0.316
Dukes D ($n=24$)	2.9167 ± 0.6339	4.8333 ± 0.6959	0.033	2.2917 ± 0.5630	3.6250 ± 0.6963	0.156
P	0.633	—	—	0.783	—	—

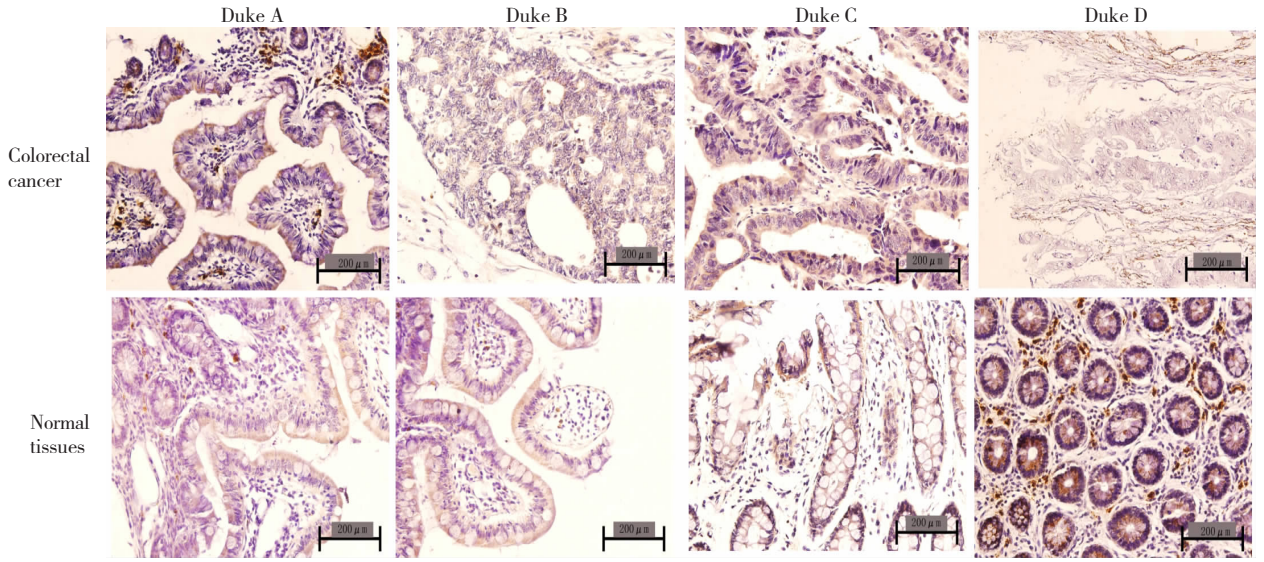

Fig. 3 Expressions of *NGX6-S* in colorectal cancer tissues and paired normal tissues of 4 stages colorectal cancer.

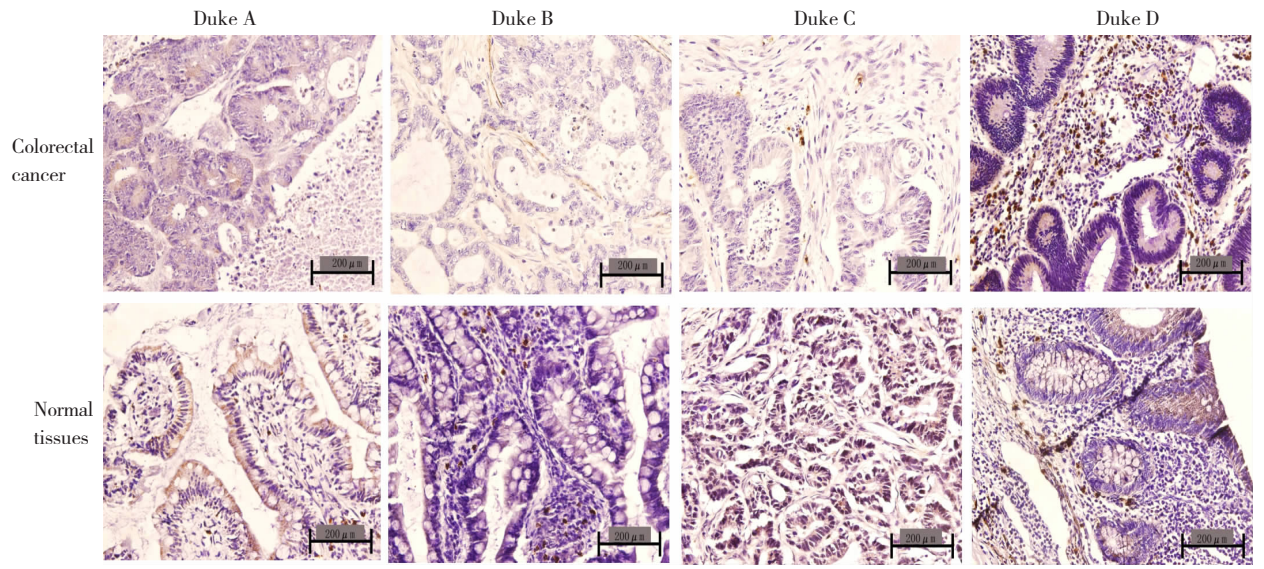

Fig. 4 Expression of *NGX6-L* in colorectal cancer tissues and paired normal tissues of 4 stages colorectal cancer.

2.1.3 Relationship between *NGX6-S*, *NGX6-L* and distant metastasis of colorectal cancer

One hundred cases of colorectal cancer were divided into a distant metastases group and a non-distant metastases group. The expression of *NGX6-S* in cancer tissues of the non-distant metastases group was higher than that in the paired normal tissues ($P = 0.043$); and that in cancer tissues of the distant me-

tastases group was lower than that in the paired normal tissue ($P = 0.033$), while the expression of *NGX6-S* in the distant metastases group and the non-distant metastases group was not different ($P = 0.360$). There was not different in expression of *NGX6-L* in the colorectal cancer tissues and the paired normal tissues in these 2 groups, respectively ($P = 1.000$, $P = 0.156$), and the expression of *NGX6-L* in colorectal cancer tissues of these 2 groups was also not different ($P = 0.456$, Tab. 2).

Tab. 2 The expression of *NGX6-S* and *NGX6-L* in colorectal cancer and paired normal tissues of the cases with or without distant metastasis ($n = 100$)

Groups	*NGX6-S*			*NGX6-L*		
	No distant metastasis ($n = 76$)	Distant metastasis ($n = 24$)	*P*	No distant metastasis ($n = 76$)	Distant metastasis ($n = 24$)	*P*
Normal tissues	2.7500 ± 0.3383	4.8333 ± 0.6959	—	2.7368 ± 0.3181	3.6250 ± 0.6963	—
Colorectal cancer	3.5867 ± 0.3465	2.9167 ± 0.6339	0.360	2.7733 ± 0.3041	2.2917 ± 0.5630	0.456
P	0.043	0.033	—	1.000	0.156	—

2.2 Correlation analysis of expression of *NGX6-S* and *NGX6-L* with carcinoembryonic antigen in the colorectal cancer

There was no correlation between the expression of *NGX6-S* or *NGX6-L* in the cancer tissues and the serum CEA ($r_s = -0.169$, $P = 0.175$; $r_s = -0.012$, $P = 0.922$, respectively). Further, there was no correlation between *NGX6-S* and serum CEA in Duck D stage ($r_s = -0.218$, $P = 0.385$).

3 DISCUSSIONS

Recently our group discovered that *NGX6* gene had 3 kinds of transcripts. *NGX6-1* and *NGX6-2* translated the same kind of protein, called *NGX6-L*; while *NGX6-3* translated another kind of protein, called *NGX6-S*. Different transcripts of gene may have different biological functions; for instance, tumor suppressor gene *ATBF1* (AT motif binding factor 1), an α-fetoprotein (AFP) transcription regulating factor, has 2 kinds of transcripts: *ATBF1-A* and *ATBF1-B*. These 2 kinds of transcripts have opposite effect on the regulation of AFP expression; the *ATBF*1-*A* has tumor-inhibition functions, and *ATBF1-B* can promote the cancer cell proliferation[8]. Among the 3 transcripts of *RASSF1* gene, *RASSF1-A* has the tumor suppression function, while the other 2 transcripts *RASSF1-B* and *RASSF1-C* are expressed ubiquitous-ly[9-10]. Different transcripts of genes as *hASIP*, *FHIT* and *WT1* have different biological functions in the tumor[11-15]. But how are the functions of 2 transcripts of *NGX6* gene in colorectal cancer?

In this study, we firstly analyzed the expression pattern of 2 transcripts of *NGX6*: *NGX6-S* and *NGX6-L* in colorectal cancer tissues and paired normal tissues of different stages, and discovered that the short transcript *NGX6-S* was the main expression form of NGX6 gene in the colorectal cancer; and there was no significant difference of expression of *NGX6-S* in the colorectal cancer tissues of different stages, and the expression of *NGX6-L* in colorectal cancer tissues of different stages was also not different; and there was no difference of expression of *NGX6-S* in the colorectal cancer tissues of Duke A, B and C stages, compaired with the paired normal tissues, while the expression of *NGX6-S* in the colorectal cancer tissues of Duke D stage was lower than that in the normal paired tissues, suggesting that the downregulation of *NGX6-S* was correlated with the distant metastasis of colorectal cancer.

To clarify the relationship of 2 transcripts of *NGX6* gene with distant metastasis of colorectal cancer, we divided 100 colorectal cancers into the non-distant metastasis group and the distant metastasis group. There was no significant difference of the expression of *NGX6-S* in the cancer tissue between the non-distant metastasis group and the distant metastasis group. The expression of *NGX6-S* in the colorectal cancer without distant metastasis was higher than that in paired normal tissues; and the expression of *NGX6-S* in the colorectal cancer tissues with distant metastasis was lower than that in the normal paired tissues. These results suggested that the downregulation of *NGX6-S* was correlated with the distant metastasis of colorectal cancer. The distant metastasis of tumor is mainly through the way of blood metastasis, which is related to the adhesion ability of the tumor cells, tumor angiogenesis, and matrix hydrolysis [16-19]. Therefore, it is inferred that *NGX6* may affect these biological processes and inhibit distant metastasis of tumor cells. *NGX6* gene could inhibit the ability of tumor cell adhesion and angiogenesis, which was discovered in our preliminary study; indicating that *NGX6-S* was an advanced molecular event for the tumorigenesis and development of colorectal cancer, and it was expected to become a molecular marker of distant metastasis of colorectal cancer.

CEA is a serum marker of colorectal cancer in the advanced stage[20], which plays an important role in the diagnosis, efficacy judgment, and recurrence judgment of colorectal cancer. In this study, the correlation between *NGX6-S*, *NGX6-L* and serum CEA was analyzed, and the 2 transcripts of *NGX6* have no correlation with serum CEA. The further analysis of the correlation between *NGX6-S* and serum CEA in cases with Dukes D stage colorectal cancer was carried out and it was confirmed that there was no correlation.

In summary, the main expression form of self-cloned candidate tumor suppressor gene *NGX6* is *NGX6-S*; and its low expression is correlated with distant metastasis of colorectal cancer, which is expected to become a treatment target of distant metastasis of colorectal cancer. Expression of *NGX6-L* is generally low in the colorectal cancer tissues and normal paired tissues, which may have no tumor inhibitory function. This study provided a valuable experimental evidence for the tumor inhibitory mechanism of *NGX6* gene.

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