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World J Gastroenterol 2004;10(9):1361-1364
World Journal of Gastroenterology
 ISSN 1007-9327

• BRIEF REPORTS •

Expression of tumor related gene NAG6 in gastric cancer and restriction fragment length polymorphism analysis

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Supported by the atural Science Foundation of Hunan Province, No.02JJY2049 and the National "863" Program of China, No.102-10-01-05
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Received: 2003-04-12 **Accepted:** 2003-09-18

Abstract

AIM: NAG6 gene is a novel tumor related gene identified recently. This study was designed to examine the expression of this gene in gastric cancer and corresponding normal tissues, and to investigate its role in the occurrence and development of gastric cancer, also to study if the genetic structure of NAG6 was altered in gastric cancer.

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR), Northern blot analysis and dot hybridization were used to compare the expression level of NAG6 gene in 42 cases of gastric cancer tissues with their corresponding normal tissues of the same patients respectively. In addition, restriction fragment length polymorphism (RFLP) analysis was adopted to study if the genetic structure of NAG6 was altered in gastric carcinomas.

RESULTS: The expression of NAG6 in 57.1% gastric cancer tissues (25/42) was absent by RT-PCR analysis. The down-regulation rate of NAG6 in gastric cancer tissues was significantly higher than that in corresponding normal tissues ($P<0.01$). However no correlation between the down-regulation of NAG6 and lymph-node and/or distance metastasis was found in this study ($P>0.05$). Dot hybridization confirmed the results of RT-PCR. Furthermore, the results of *Eco*RI RFLP analysis of NAG6 gene demonstrated that 3 of 7 cases of gastric cancer showed loss of 5 kb fragment in comparison with their corresponding normal tissues.

CONCLUSION: NAG6 gene is significantly down regulated in gastric cancer. The loss of genetic materials may be the cause of down-regulation of NAG6 expression. This seems to suggest that NAG6 may represent a candidate of putative tumor suppressor gene at 7q31-32 loci associated with gastric carcinoma. The down-regulation of this gene may play a role in occurrence and development of this disease, however it may not be associated with lymph node and/or distance metastasis.

Zhang XM, Sheng SR, Wang XY, Bin LH, Wang JR, Li GY. Expression of tumor related gene NAG6 in gastric cancer and restriction fragment length polymorphism analysis. *World J Gastroenterol* 2004; 10(9): 1361-1364
http://www.wjgnet.com/1007-9327/10/1361.asp

INTRODUCTION

Gastric cancer (GC) is one of the leading causes of cancer death in the world, although its incidence has gradually declined in recent years[1,2]. However, in the Far East, including China and Japan, gastric cancer remains a prevalent cancer with a high mortality[3,4]. It is well known that carcinogenesis and progression of human gastric cancer are related to multiple genetic aberrations including activation of oncogenes and inactivation of tumor suppressor genes. The latter involves the loss of heterozygosities (LOH) of several chromosomal loci and mutations in tumor suppressor genes, such as *p53* and *DCC* genes. However, the mechanism of the process of multistage carcinogenesis is still not well understood[5-12]. Recently, a number of cytogenetic and molecular genetic studies have revealed that LOH on the long arm of chromosome 7 occurs frequently in many types of primary cancers including nasopharyngeal, gastric, breast, ovarian, and oral carcinomas, and investigators have identified the most common site of LOH as 7q31-32, implying the existence of at least one multi-tissue tumor suppressor gene (TSG) at this locus[13-22]. Based on these findings, in our previous studies, we have cloned a novel tumor related gene from this common deletion region in 7q31-32 by positional candidate cloning strategy, we named it NAG6, and its GenBank accession number was AF156971. It was found to be a potential tumor suppressor gene associated with NPC[23-26]. To investigate whether the expression of NAG6 was also altered in GC and whether NAG6 gene also played a role in the pathogenesis of gastric carcinoma, we analyzed the expression level of NAG6 in 42 cases of human gastric carcinoma and their matched normal tissues by RT-PCR, Northern blot analysis and dot hybridization. Furthermore, to study if the genetic structure alteration of NAG6 was the reason of its abnormal expression in GC, RFLP analysis was adopted. These studies can lead to a better understanding of the molecular mechanism of gastric cancer.

MATERIALS AND METHODS

Tumor specimens

Fresh surgical specimens of forty-two gastric carcinoma (GC) and corresponding normal tissues were obtained from the Affiliated Xiangya Hospital of Central South University from January 2000 to July 2000. All tumor specimens were confirmed by pathological diagnosis. Each freshly resected specimen was frozen immediately and stored in liquid nitrogen until analyzed. Histologically, In the 42 cases of gastric carcinoma, 4 were well-differentiated adenocarcinomas, 30 poorly-differentiated adenocarcinomas, 6 signet ring cell carcinomas and 2 mucoid carcinomas. There were 22 males and 20 females, their age ranged from 30 to 68 years (mean

age, 51.7 years). Six cases had lymph node or distance metastases. No patient had received chemotherapy or radiation therapy before surgery.

RT-PCR

Total RNA was isolated using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA) according to the protocol provided by the manufacturer. After treated with DNase-I (Promega), 1-2 μg of total RNA was reversely transcribed into complementary DNA (cDNA) with oligo(dT) using cDNA synthesis kit (Promega). Then 1 μL product was used as the template to amplify specific fragments in a 25 μL reaction mixture. The subsequent PCR was performed using Taq polymerase and the buffer (Promega) supplied with 0.2 mmol/L dNTPs and 0.2 μmol/L primers. Primers corresponding to NAG6 sequences were designed with WWW Primer Picking (Primer 3) and synthesized by TaKaRa. Gene-specific forward and reverse primers for NAG6 were designed to produce a PCR product of 680 bp. RT-PCR reaction was carried out with an initial denaturation at 95 ℃ for 5 min, followed by 35 cycles at 94 ℃ for 50 s, annealing at 56 ℃ for 50 s, at 72 ℃ for 60 s, and a final extension at 72 ℃ for 10 min. At the same time, a housekeeping gene, GAPDH was amplified as internal control to normalize the relative levels of cDNA, which generated a PCR product of 475 bp. An aliquot (10 μL) of each reaction product was analyzed by 10 g/L agarose gel electrophoresis.

The sequences of primers were as follows: NAG6F1, 5'-GGCACTGGAGTACAAAGACA-3'; NAG6R1, 5'-TTACTTTTCCCATTTGCTCA-3'; GAPDHF1, 5'-GTCATCCATGACAACTTTGGTATC-3'; GAPDHR1, 5'-CTGTAGCCAAATTCGTTGTCATAC-3'.

Northern blot analysis

Total RNA was isolated from human gastric carcinoma and corresponding normal tissues by Trizol reagent (Gibco-BRL), and hybridization was performed as described. A 30 μg RNA was separated by electrophoresis by denaturing agarose gels and blotted onto nylon membrane (Clontech). RNA was permanently attached to the membrane by UV illumination for 150 s (GS Gene Linker, Bio-Rad, USA), and the membrane was dried in a vacuum at 80 ℃ for 2 h and sealed in a plastic bag for use. The hybridization probes were obtained by RT-PCR amplification. NAG6 cDNA probe was random-prime labeled with [α-^{32}P]dCTP using primer-a-gene random labeling kit (Promega, USA) and following the protocol. Hybridization with the RNA blots was carried out at 68 ℃ overnight in Express Hyb TM hybridization solution (Clontech) in a rolling bottle. The membranes were washed twice at room temperature in 2×saline sodium citrate (SSC), 0.5 g/LSDS for 10 min, once at 42 ℃ in 1×SSC, 1 g/L SDS for 15 min and once at 50 ℃ in 0.1×SSC, 1 g/L SDS for 30 min. Then, they were exposed to film (Eastman Kodak, Rochester, NY, USA) for 4 d at -70 ℃. After exposure, the blot was again hybridized with a GAPDH probe.

Dot blot analysis

GAPDH and NAG6 cDNA fragments containing open reading frames from cDNA of gastric carcinoma samples were obtained by RT-PCR. These cDNAs were reclaimed and purified by using a kit according to the instructions of its manufacturer (Shanghai Huashun Co.). After alkali dissolution, GAPDH and NAG6 cDNA were blotted onto nylon membranes. cDNA was permanently attached to the membrane by UV illumination for 150 s, and the membranes were dried in a vacuum at 80 ℃ for 2 h to fix the cDNA. Ten μg total RNA was isolated from 10 cases of human gastric carcinomas and 10 cases of each corresponding normal tissues, and reversely-transcribed into cDNA probe with oligo (dT) and [α-^{32}P] dCTP using cDNA synthesis kit after treated with DNase I and RNasin at 37 ℃ for 1 h to remove contaminated DNA (1 μg total RNA of each case was used). Then the two cDNA probes were hybridized with GAPDH and NAG6 cDNA blots respectively as Northern hybridization described above.

Southern-based RFLP analysis

Genomic DNA was extracted from gastric cancer and corresponding normal tissues by using sodium dodecyl sulphate (SDS), EDTA, proteinase K, dispelled protein and phenol-chloroform methods, removing RNA with RNA enzyme, precipitating DNA with alcohol of two times in volume, mixed in proper TE buffer solution, and kept for use at 4 ℃. Genomic DNA was digested with the stated restriction endonuclease *Eco*RI and electrophoresed on 7 g/L agarose (TAE) gel. After electrophoresis, DNAs were denatured, neutralized and transferred to nylon membranes. Then DNAs were permanently attached to the membrane by UV illumination and the membrane was dried in a vacuum at 80 ℃ for 2 h. The nylon membrane was hybridized with the radiolabelled NAG6 cDNA probe according to the method of Southern blot. After washed and autoradiographed at - 70 ℃ for 3 to 5 d, hybridizing was carried out.

Statistical analysis

Chi-square test was used. A P value less than 0.05 was considered statistically significant.

RESULTS

Expression of NAG6 in gastric cancer and corresponding normal tissues

In 42 pairs of GC and corresponding normal tissues, NAG6 expression was undetectable in 24 tumors (57.1%), while it was detectable in all corresponding normal tissues. The expression of NAG6 in gastric carcinomas was significantly down-regulated than that in normal tissues (χ^2=33.6, P<0.005). Representative cases of NAG6 expression detected by RT-PCR are shown in Figure 1. The down-regulation rate of NAG6 in patients with lymph node and/or distance metastases and those without lymph-node and/or distance metastases was 66.7%(4/6) and 47.2%(17/36) respectively. There was no apparent relevance between NAG6 down-expression and lymph node and/or distance metastasis of gastric carcinomas (P>0.05).

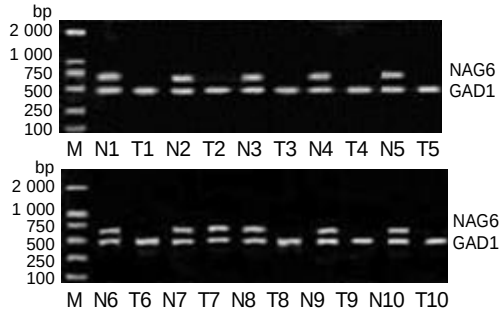

Figure 1 Expression of NAG6 in gastric carcinoma and corresponding normal tissues examined by RT-PCR. The RT products were examined by PCR with NAG6 primers, producing a 680 bp fragment and with GAPDH primers, producing a 466 bp fragment. Lane M: 2 000 bp marker, Lane N: normal epithelium tissues, Lane T: gastric carcinoma tissues.

In order to verify the results of RT-PCR, Northern hybridization was performed. Northern blot analysis did not detect NAG6 expression in both gastric carcinoma and

corresponding normal tissues, whereas GAPDH was strongly expressed in both of them. We speculated that the expression abundance of NAG6 gene in gastric cancer and corresponding normal tissues might be too low to be detected by Northern blot analysis. So, we used dot hybridization analysis to verify the reliability of RT-PCR on the other hand. The results of dot hybridization confirmed the results of RT-PCR that the expression of NAG6 was significantly down-regulated in gastric carcinoma tissues (Figure 2).

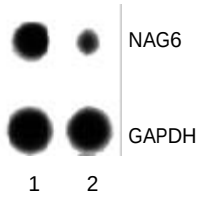

Figure 2 Dot hybridization analysis of NAG6 gene expression profiles in human gastric carcinoma and corresponding normal tissues. NAG6 cDNA obtained by RT-PCR was blotted onto nylon membranes. The membranes were hybridized with ^{32}P-labeled cDNA probes obtained from total RNA of human gastric carcinoma (1) and corresponding normal gastric epithelial (2) tissues. After stringent washes, membranes were exposed to X-ray film for 4 d at-70 ℃. NAG6 was down-regulated in gastric carcinoma tissues.

RFLP analysis

*Eco*RI RFLP analysis of NAG6 gene was performed in 7 cases of gastric cancer and corresponding normal tissues. The results showed that there were two kinds of common allelic fragments (11.5 Kb, 5.0 Kb) in all corresponding normal tissues and 4 cases of gastric cancer, but 3 cases of gastric cancer tissues showed loss of 5 Kb fragment in comparison with their matched normal tissues.

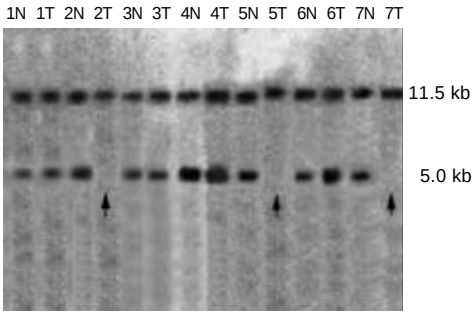

Figure 3 RFLP analysis using Southern hybridization. Gastric cancer and normal epithelium genome DNAs were digested with *Eco*RI and hybridized with NAG6 cDNA probe. Three cases of gastric cancer tissues showed loss of 5Kb fragment (N: normal epithelium tissues, Lane T: gastric carcinoma tissues).

DISCUSSION

NAG6 gene has been recently identified and cloned by our group at chromosome 7q31-32, the common deletion site in various human malignancies. Comparison with GenBank and EMBO database using the BLAST program the cDNA sequence of NAG6 gene was a unique gene with no homology to any previously reported human genes, and its GenBank accession number is AF156971. The predicted NAG6 protein contained four protein kinase C (PKC) phosphorylation sites, suggesting that the activity of NAG6 protein can be regulated by phosphorylation. Its mRNA expression level in NPC biopsies was significantly lower than that in normal nasopharyngeal epithelium, and the down-regulation of NAG6 in NPC was attributable to several factors including loss of genetic materials and hypermethylation. All these findings supported NAG6 as a candidate tumor suppressor gene at 7q31-32. The down-regulation of this gene might play a role in occurrence and development of NPC[23-26].

Cytogenetic and molecular analyses demonstrated that frequent LOH on the long arm of chromosome 7 could also be observed in a high proportion of gastric cancer cases[13-16]. Nishizuka *et al* reported LOH at any locus on 7q occurred in 34% (18 out of 53) of primary gastric carcinomas[14]. Kuniyasu *et al* examined LOH on the long arm of chromosome 7 using 5 polymorphic marker probes in 98 gastric carcinomas[15]. The results showed twenty-six of 82(32%) informative cases showed LOH on 7q at least one locus of 5 loci. Xia *et al* studied a total of 28 primary gastric cancer specimens, and they found that deletion of 7q (21/26) was one of the characteristic structural changes of primary gastric cancer[16]. Furthermore, investigators have identified the most common site of LOH as 7q32-qter, and concluded that in the 7q32-qter segments, at least one tumor suppressor gene probably existed and it might have a close relation to the development and progression of gastric cancer[13-16]. NAG6 gene located at 7q31-32 locus. We were interested in whether expression of NAG6 was altered in GC and whether NAG6 was also a possible tumor suppressor in human gastric carcinoma. In this study, RT-PCR, Northern blot and dot hybridization were used to detect the expression abundance of the gene in gastric carcinoma and corresponding normal tissues. The results of RT-PCR showed that the down-regulation rate of NAG6 in gastric carcinoma tissues was significantly higher than that in corresponding normal tissues (P<0.005). Dot hybridization confirmed the results of RT-PCR. However the expression of NAG6 was not relevant to lymph node and/or distance metastasis of gastric carcinomas. This seems to suggest that down-regulation of NAG6 might play a role in the occurrence and progression of GC.

In order to study the possible cause of down-regulation of NAG6 in gastric cancers, we studied on the restriction fragment length polymorphisms (RFLPs) of NAG6 gene in gastric cancer and corresponding normal tissues to detect if the genetic structure of NAG6 was changed in GC. We used restriction enzymes to cut DNA at specific recognition sites, fragments of restricted DNA separated by gel electrophoresis and detected by subsequent Southern blot hybridization to a radiolabeled DNA probe. In recent twenty years, the application value of restriction fragment length polymorphism (RFLP) analysis in the detection of genetic structure change and genetic polymorphisms of candidate gene has called attention of the scholars at home and abroad[27-29]. Polymorphic sequences that result in RFLPs are used as markers on both physical maps and genetic linkage maps. In this study, *Eco*RI RFLP analysis of NAG6 gene was performed in 7 cases of gastric cancer and corresponding normal tissues. The results demonstrated that 3 of 7 cases of gastric cancer showed loss of 5 kb fragment in comparison with their corresponding normal tissues. In the previous study, RFLP analysis also found that 6 of 14 NPC cases lost the fragment of 3 kb in comparison with their matched peripheral blood lymphocytes. These results demonstrated that the genetic structure of NAG6 was changed in both NPC and GC. A preliminary conclusion was drawn that loss of genetic materials might be the cause of down-regulation of NAG6 expression.

To summarize, our data showed that NAG6 was down-regulated in gastric cancer, and loss of genetic materials of NAG6 was also found in GC. It is reasonable to predict that NAG6 may represent a candidate of putative tumor suppressor gene at 7q31-32 locus associated with GC and NPC, and this gene may play an important role in suppressing GC tumorigenesis, losses of its function may contribute to the occurrence and

development of GC. The mechanism of this gene is still unclear. Further studies on a large patient population are needed to verify these initial observations and to characterize the mechanism of down-regulation of NAG6 in tumors. It is important to examine the possible relationship between loss or preservation of NAG6 expression and clinical outcome in patients with tumor.

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Edited by Zhang JZ and Wang XL **Proofread by** Xu FM