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

Xiao-Mei Zhang, Shou-Rong Sheng, Xiao-Yan Wang, Liang-Hua Bin, Jie-Ru Wang, Gui-Yuan Li

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 EcoRI 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.

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 56±13.55 years).
age, 51.7 years). Six cases had lymph node or distant 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 primers for NAG6 were designed with the RNA blots was carried out at 68°C for 1-2 min. The PCR reaction was carried out with an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 50 s, annealing at 56°C for 50 s, and a final extension at 72°C 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% agarose gel electrophoresis.

The sequences of primers were as follows: NAG6F1, 5’-GCCACTGGGAGTACAAGAACA-3’; NAG6R1, 5’-TTACTTTTCCATTTGGCTCA-3’; GAPDHF1, 5’-GTCATCCATGACAACTTTGGTATC-3’; GAPDHR1, 5’-CTGTAAGCCAATTCGTTGTACATC-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. 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 0.1×SSC, 1 g/L SDS, dispelling protein and removing RNA with RNA enzyme, precipitating DNA with alcohol of two times in 0.1×SSC, 1 g/L SDS, and RNA with RNA enzyme, precipitating DNA with alcohol of two times in 0.1×SSC, 1 g/L SDS. Genomic DNA was digested with the stated restriction endonuclease EcoRI and electrophoresed on 7% 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°C 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°C 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 (χ²=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 distant 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 ^32P-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 °C. NAG6 was down-regulated in gastric carcinoma tissues.

**RFLP analysis**

EcoRI 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 EcoRI 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.

Cytogetic 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. Nishizuka et al reported LOH at any locus on 7q occurred in 34% (18 out of 53) of primary gastric carcinomas. Kuniyasu et al examined LOH on the long arm of chromosome 7 using 5 polymorphic marker probes in 98 gastric carcinomas. 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. 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. 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 polymorphisms (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. Polymorphic sequences that result in RFLPs are used as markers on both physical maps and genetic linkage maps. In this study, EcoRI 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 tissue. 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.

REFERENCES

1. Stadtlander CT, Waterbor JW. Molecular epidemiology, pathogenesis and prevention of gastric cancer. Carcinogenesis 1999; 20: 2195-2208
2. Palli D. Epidemiology of gastric cancer: an evaluation of available evidence. J Gastroenterol 2000; 35: Supplementary: 84-89
3. Maehara Y, Kakeji Y, Oda S, Takahashi I, Akazawa K, Sugimachi K. Time trends of surgical treatment and the prognosis for Japanese patients with gastric cancer. Br J Cancer 2000; 83: 986-991
4. Deng DJ. Progress of gastric cancer etiology: Nitrosamines in the 1990s. World J Gastroenterol 2000; 6: 613-618
5. Becker KF, Keller G, Hoefer H. The use of molecular biology in diagnosis and prognosis of gastric cancer. Surg Oncol 2000; 9: 5-11
6. Boussioutas A, Taupin D. Towards a molecular approach to gastric cancer management. Intern Med J 2001; 31: 296-303
7. Yasui W, Oue N, Kuniyasu H, Itô R, Táhara E, Yokozaki H. Molecular diagnosis of gastric cancer: present and future. Gastric Cancer 2003; 4: 113-121
8. Maltoni M, Volpi A, Nanni O, Bajorek P, Belletti E, Vecchi AM, Liverani M, Danesi S, Calistri D, Ricotti L, Amadori D. Gastric cancer: epidemiologic and biological aspects. Forum 1998; 8: 199-207
9. Xu AG, Li SG, Liu JH, Gan AH. Function of apoptosis and expression of the proteins Bcl-2, p53 and C- myc in the development of gastric cancer. World J Gastroenterol 2001; 7: 403-406
10. Liu LX, Liu ZH, Jiang HC, Qu X, Zhang WH, Wu LF, Zhu AL, Wang XQ, Wu M. Profiling of differentially expressed genes in human gastric carcinoma by cDNA expression array. World J Gastroenterol 2002; 8: 580-585
11. Meltzer SJ. Tumor genomics vs tumor genetics: a paradigm shift? Gastroenterology 2001; 121: 726-729
12. Nishizuka S, Tamura G, Terashima M, Satodate R. Loss of heterozygosity during the development and progression of differentiated adenocarcinoma of the stomach. J Pathol 1998; 185: 38-43
13. Zenklusen JC, Conti CJ. Cytogenetic, molecular and functional evidence for novel tumor suppressor genes on the long arm of human chromosome 7. Mol Carcinog 1996; 15: 167-175
14. Nishizuka S, Tamura G, Terashima M, Satodate R. Commonly deleted region on the long arm of chromosome 7 in differentiated adenocarcinoma of the stomach. Br J Cancer 1997; 76: 1567-1571
15. Kuniyasu H, Yasui W, Yokozaki H, Akagi M, Akama Y, Kitahara K, Fujii K, Táhara E. Frequent loss of heterozygosity of the long arm of chromosome 7 is closely associated with progression of human gastric carcinomas. Int J Cancer 1994; 59: 597-600
16. Xia J, Xiao S, Zhang J. Direct chromosome analysis and FISH study of primary gastric cancer. Zhonghua Zhongliu Zazhi 1999; 21: 345-349
17. Tan G, Xiao J, Tian Y, Dong L, Jiang N, Zhan F, Li G. Microsatellite analyses of loci at 7q31.3-q36 reveal a minimum of two common regions of deletion in nasopharyngeal carcinoma. Otalaryngol Head Neck Surg 2002; 126: 296-300
18. Zenklusen JC, Weintrub LA, Green ED. Construction of a high-resolution physical map of the approximate 1-Mb region of human chromosome 7q31.1-q32 harboring a putative tumor suppressor gene. Neoplasia 1999; 1: 16-22
19. Lin JC, Scherer SW, Tougas L, Traverso G, Tsai LC, Andrusis IL, Johy S, Park M. Detailed deletion mapping with a refined physical map of 7q31 localizes a putative tumor suppressor gene for breast cancer in the region of MET. Oncogene 1996; 13: 2001-2008
20. Koike M, Takeuchi S, Yokota J, Park S, Hatta Y, Miller CW, Tsuokuma N, Koeffler HP. Frequent loss of heterozygosity in the region of the D7S523 locus in advanced ovarian cancer. Genes Chromosomes Cancer 1997; 19: 1-5
21. Koike M, Tasaka T, Spira S, Tsuoruka N, Koeffler HP. Allelotyping of acute myelogenous leukemia: loss of heterozygosity at 7q31.1 (D7S486) and q33-34 (D7S498, D7S505). Leuk Res 1999; 23: 307-310
22. Wang XL, Uzawa K, Miyakawa A, Shiiba M, Watanabe T, Sato T, Miya T, Yokoe H, Tanazawa H. Localization of a tumour-suppressor gene associated with human oral cancer on 7q31.1. Int J Cancer 1998; 75: 671-674
23. Jiang N, Zhan F, Tan G, Deng L, Zhou M, Cao L, Qiu Y, Xie Y, Li G. A cDNA located on chromosome 7q32 shows loss of expression in epithelial cell line of nasopharyngeal carcinoma. Chin Med J 2000; 113: 650-653
24. Jiang N, Zhan F, Xie Y, Zheng Z, Zhou M, Deng L, Li G. Establishment of partial gene expression map of 7q32 in nasopharyngeal carcinoma and primary culture normal nasopharyngeal epithelial cells. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 1998; 15: 267-270
25. Jiang N, Deng LW, Tan GL, Zhan FH, Zhou M, Cao L, Qiu YZ, Xie Y, Li GY. A nasopharyngeal carcinoma negatively related EST on 7q32. Yichuan Xuebao 1999; 26: 301-308
26. Zhang XM, Sheng SR, Wang XY, Xiang Q, Li J, Tan C. Expression of tumor related gene NAG6, NAG-7, BRD7 in gastric cancer. Zhonghua Xiu Hua Za Zhi 2002; 22: 733-736
27. Yuan Y, Dong M, Lu P, Wang XJ, Jin CL, He AG. Restriction fragment length polymorphism of pepsinogen C gene in patients with stomach carcinoma and in its high risk population. China Natl J Natl Gastroent 1996; 2: 223-225
28. Byrne M, Parish TL, Moran GF. Nuclear RFLP diversity in Ecaecylus nitens. Heredity 1996; 81: 225-223
29. Butcher PA, Moran GF, Perkins HD. RFLP diversity in the nuclear genome of Acacia mangium. Heredity 1998; 81: 205-213

Edited by Zhang JZ and Wang XL Proofread by Xu FM