Role of the *nm23-H1* Gene in the Metastasis of Gastric Cancer

The *nm23* gene is now generally accepted as one of the suppressor genes for metastasis in many types of human cancer. To investigate the role of the *nm23* gene in gastric cancer, we examined the expression of nm23-H1 mRNA, mutations, and loss of heterozygosity (LOH) of the *nm23-H1* gene in gastric cancers. The expression of nm23-H1 mRNA was examined by Northern blot analysis in eight paired sets of specimens. The expression was higher in primary cancer specimens and metastatic lymph nodes than their corresponding normal gastric mucosa in all eight sets of specimens examined, while it was similar between primary cancer specimens and metastatic lymph nodes. The mutations of the *nm23-H1* gene were examined in an additional 11 sets of specimens, including eight sets of analysed by Northern blotting, by polymerase chain reaction single-strand conformation polymorphism analysis, no mutation being found in any of the 11 sets of specimens tested. LOH of the *nm23-H1* gene was also examined in additional 12 sets of specimens, among which seven (58%) specimens were informative for LOH. LOH was identified in one (14%) out of these seven informative sets. These results suggest that *nm23* may not be the metastasis suppressor gene and the alteration of this gene not play an important role in the process of metastasis of gastric cancer.
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Key Words : *Gastrointestinal neoplasms; Stomach neoplasms; Neoplasm metastasis; Blotting, Northern; Heterozygote detection; Genes, Suppressor, Tumor; Polymerase chain reaction*

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INTRODUCTION

The *nm23* gene was identified by differential hybridization of murine K-1735 melanoma subline with high metastatic potential and that with low potential (1).

The reduced expression of *nm23* mRNA and its product, nucleoside diphosphate (NDP) kinase, has been reported to be strongly associated with increased metastatic activity in many types of human cancers, such as breast, colon, hepatocellular and prostate cancer (2-6). In addition, the transfection of *nm23* cDNA into highly metastatic murine melanoma cells resulted in a significant decrease in metastatic potential, suggesting that there was an inverse relationship between *nm23* gene expression and metastatic potential (7). On the basis of these results, the *nm23* gene is now considered to be one of the suppressor genes for metastasis, although conflicting results have been reported in certain types of cancer, such as breast and colon cancer (8-10).

Recently NDP kinase has been found to have two isotypes, NDP kinase A and B, which are encoded by *nm23-H1* and *nm23-H2* gene, respectively (11, 12). Previous investigations have strongly suggested that the *nm23-H1* gene is closely related to the metastatic behavior of cancer, and the *nm23-H2* gene is reported to the differentiation of cancer cells (6, 13).

Gastric cancer is the leading cause of cancer death in Korea. However, the mechanisms of metastasis in gastric cancer have not been elucidated in detail yet. Concerning the expression of the *nm23-H1* gene in gastric cancer, a few reports have been published, demonstrating that the reduced expression of *nm23* gene is associated with increased metastasis (14, 15). However, there have been no reports, on the genetic alteration of the *nm23-H1* gene in Korean patients with gastric cancer. This study was undertaken to investigate the role of the *nm23-H1* gene by Northen blot, Southren blot and PCR-SSCP analysis in the metastasis of gastric cancer.

MATERIALS AND METHODS

Specimens

Twenty-three sets of specimens were collected from primary gastric cancer tissues and metastatic lymph

Table 1. The oligonucleotide primers used to amplicate the portions of the *nm23-H1* gene in polymerase chain reaction

Exon	Size	Position	Upstream	Downstream
Exon 1	79	15- 94	GTCTGAAAAACGTAGCGCCGG	CTTAGGTTTGAACTCCGGCTG
Exon 2	130	95-224	GCTTGAGACGGATGACGCTGTA	CAGGTTAATCACAGTGTTCTCC
Exon 3	101	225-326	ATGTCCTTAGATGGTTTGGGGGT	TTTGGTCTCATTCATGGCTGTAT
Exon 4	113	327-439	GCCACATTTTCTGCTGTGATT	CCCAAATCCTTGTGGCAACT
Exon 5	115	440-554	GTCTAATGTCCATGGAGCTTC	CAGATGGTCGGGGATGGTAAC

nodes along with uninvolved gastric tissues during operation, from 23 patients with gastric adenocarcinoma receiving gastrectomies at the Korea Cancer Center Hospital. All the tissue specimens obtained were immediately frozen in liquid nitrogen and stored at -70℃ until experiment.

Northern Blot analysis

After the pulverization of frozen tissues, total cellular RNAs were prepared by guanidine thiocyanate-phenol-chloroform extraction method. For Northern blot analysis, 20 μg of extracted RNA was denatured, electrophoresed on an agarose gel containing formaldehyde and transferred on to a Hybond nylon membrane (Amersham, UK) in 10x standard saline citrate (SSC) overnight. After immobilization with UV light (2400 UV cross-linker, Stratagene, La Jolla, CA, USA), prehybridization and hybridization reactions were performed in 50% (v/v) formamide, 5×SSC, 50 mM Tris-HCl pH 7.5, 5×Denhardts' solution, 5% (w/v) sodium dodecyl sulfate (SDS), 100 μg/ml denatured salmon sperm DNA and 10% (w/v) dextran sulfate at 42℃. The cDNA probes used were derived from the *Bam*H I-*Eco*R I fragment of pBSK- HI and labeled with [α-^{32}P] dCTP by random priming with the Megaprime DNA labeling system (Promega) (16). Blots were washed in 0.1x SSC and 0.1% (w/v) SDS at 65℃ before autoradiography at -70℃ with intensifying screens. The expression level of glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard to correct the variations caused by the amount of mRNA loaded.

Southern Blot analysis

High molecular weight DNA was extracted according to the established procedures (17). In brief, DNAs were extracted by the treatment of specimens with SDS-proteinase K, phenol, chloroform and isoamyl alcohol. Twenty μg of genomic DNA was digested with *Bgl* II, subjected to electrophoresis on 0.8% agarose gels and blotted onto a Nylon membrane. Southern hybridization, washing and autoradiography were performed the same as for Northern blot analysis as described above.

PCR-SSCP analysis

Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis was performed as described by Hong et al. (18) with minor modifications. The oligonucleotides were synthesized by the phosphoroamidite method using a DNA synthesizer 391 PCRMATE (Applied Biosystems, CS, USA) and purified by high performance liquid chromatography and reverse chromatography. The sequences of primers used for the detection of mutations of the nm23-H1 gene were the same as reported by Bafico et al. (19) (Table 1).

PCR was performed in a 50 μl of reaction mixture containing 100 ng of genomic DNA, 20 pmol of a primer, 200 mM of dATP, dCTP, dGTP and dTTP, 0.1 mCi of [α-^{32}P] dCTP (3,000 Ci/nmol) (Amersham) and 0.5 U of Taq polymerase (Perkin Elmer Cetus, USA). The five sets of oligonucleotides used as primers for PCR were amplified DNAs corresponding to the codons for exon 1-5 with flanking intron sequence by the reaction of 35 cycles. Each cycle consisted of denaturation at 94℃ for 1 min, annealing at 62℃ for 1 min and extension at 72℃ for 1 min by using a DNA Thermal cycler (Perkin Elmer, USA). After amplification, one tenth of the final product was mixed 1:1 with 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. After denaturation by heat, electrophoresis was performed on 6% polyacrylamide gel with or without 10% glycerol. The gel was dried on a filter paper and autoradiography was done.

RESULTS

nm23-H1 mRNA expression

The expression of *nm23-H1* mRNA was examined by Northern blot analysis in eight paired sets of specimens in which all the sets of specimens showed higher expression of the mRNA in primary cancer and metastatic lymph nodes compared with their corresponding normal

Fig. 1. nm23-H1 mRNA expression by Northern blot analysis. L/N indicates lymph node metastatic tumor, T: primary tumor, and N: normal mucosa.

gastric mucosae. The mRNA of nm23-H1 was expressed at a similar level between primary cancers and metastatic lymph nodes in all the sets of specimens tested. Representative results are shown in Fig. 1. We also analyzed the expression of nm23-H1 mRNA according to the clinical and pathological data, resulting in no significant association of nm23-H1 RNA level with age, sex, cancer location, differentiation, lymph node metastasis or distant metastasis (data not shown).

PCR-SSCP analysis

Mutations in coding or splicing regions of nm23-H1 mRNA were examined by PCR-SSCP analysis using five kinds of primers showing in Table 1, in DNAs of 11 sets of specimens. No abnormal band was detected in any specimen of the 11 sets, including eight sets of analysed by Northern blotting, tested. The representative results of the PCR-SSCP analysis are shown in Fig. 2.

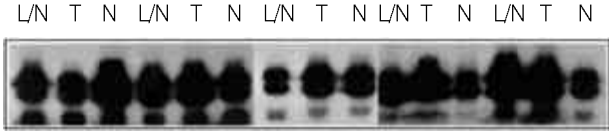

Fig. 2. PCR-SSCP analysis of *nm23-H1* gene exon 2 in a primary gastric adenocarcinoma specimen and corresponding metastatic lymph node and non-cancerous mucosa. L/N indicates lymph node metastatic tumor, T: primary tumor, and N: normal mucosa.

Incidence of LOH

To confirm the rare event of *nm23* DNA mutations, DNAs extracted from another 12 sets of specimens were examined for LOH of the *nm23-H1* gene by a *Bgl*II restriction fragment length polymorphism (RFLP) using human chromosomal DNAs which had *nm23-H1* allelic bands at 2.3 and 7.6 kb. Seven (58%) of the 12 specimens were heterozygous and LOH was observed in one (14%) out of these seven informative specimens examined. The 2.3-kb allele of the *nm23* gene was markedly reduced in cancer tissues when compared to corresponding normal mucosa (Fig. 3B), while the band of 7.6-kilobase allele was amplified. The 21-kb band was identified in all DNAs isolated from cancer tissues and normal mucosae.

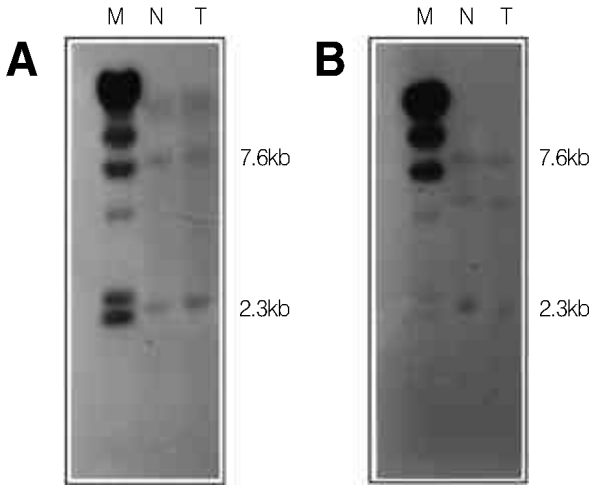

Fig. 3. Allelic loss of *nm23-H1* gene in gastric cancer. M: λ phage *Hind*III cut size marker, N: Normal mucosa, T: Primary tumor tissues.

DISCUSSION

There is increasing evidence that the *nm23* gene plays an important role in suppressing cancer metastasis. However, there is no definite evidence, as yet, for the role of the *nm23-H1* gene in the suppression of metastasis, because conflicting results have been reported in certain types of human cancers. Several investigators have described a higher level of *nm23* gene mRNA in colorectal cancer tissues than normal mucosae and increased levels of nm23-H1 protein in tissues obtained at an advanced stage in neuroblastoma (9, 20). Radinsky et al. (8) reported that the mRNA of *nm23* gene was expressed at similar levels regardless of their metastatic potential in human colon cancer cells transplanted in nude mice. An increased level of nm23-H1 expression has also been reported in both high metastatic and low metastatic colorectal carcinomas (9). No relationship between lymph node metastasis and *nm23* gene expression has been observed in breast cancer (10).

In gastric cancer, Nakayama et al. (14) and Kodera et al. (15) have reported that the decreased expression of nm23 gene played a role in metastasis. In this study, we observed high levels of the *nm23-H1* gene in both primary cancer specimens and metastatic lymph nodes and no difference in expression levels between primary cancer and metastatic lymph nodes.

For these conflicting results in gastric cancer we suggest the following possible explanations. One possibility is that *nm23-H1* gene regulation may be different between different patients and the *nm23-H1* gene may not have universally suppressive activity for metastasis (1). Another possibility is that *nm23-H1* gene expression is not related to the increased potential for metastasis but to the development of gastric cancer. The third is that the metastasis suppressor gene is not the *nm23-H1* gene but a gene linked to or in close proximity to the *nm23-H1* gene (21).

The genetic alterations of the *nm23-H1* gene have been reported to develop at both expression level and structural level (2, 7). Point mutation of the *nm23-H1* gene has been reported in childhood neuroblastoma and colorectal cancer (22, 23). However in this study, we failed to detect *nm23-H1* mutation in 11 sets of gastric carcinoma specimens by PCR-SSCP analysis, suggesting that specific point mutation of the *nm23-H1* gene may be a rare event in gastric cancer.

In conclusion, the results of the present study suggest that the genetic alteration of the *nm23-H1* gene may be a rare event and may not play an important role in the process of metastasis of gastric adenocarcinoma.

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