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Loss of Heterozygosity on the Short Arm of Chromosome 9 without p16 Gene Mutation in Gastric Carcinomas

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A putative tumor suppressor gene, p16 (*MST1*; multiple tumor suppressor 1/*CDK4I*; cyclin-dependent kinase 4 inhibitor), was isolated and mapped on the short arm of chromosome 9 (9p). The significance of p16 mutations in gastric tumorigenesis was examined by assessing p16 mutations as well as loss of heterozygosity (LOH) on 9p in 13 gastric adenomas and 45 adenocarcinomas. LOH on 9p (*IFNA*; α-interferon locus) was detected in 22% (5/23 informative cases) of differentiated adenocarcinomas, 10% (1/10) of undifferentiated carcinomas and none (0/6) of the adenomas. Although we found a sequence polymorphism at the second position of codon 99 (CGC/CAC) of the p16 in one gastric adenoma patient, no somatic mutations were detected in any of the gastric adenomas or adenocarcinomas. These results suggest that p16 mutations probably do not contribute to gastric tumorigenesis. However, these data suggest that another tumor suppressor gene on 9p (near the *IFNA* locus) may contribute to the progression of differentiated adenocarcinoma of the stomach.

Key words: Chromosome 9p — p16 gene — Gastric adenoma — Gastric adenocarcinoma

Chromosomal loss at 9p21-22 has been implicated in the genesis of several different tumors.[1-5] Recently, a putative tumor suppressor gene, p16 (*MST1/CDK4I*), was isolated from the region.[6,7] Homozygous deletions and mutations of p16 have been detected in many melanoma cell lines[6] and mutations of p16 have frequently been found in surgically resected squamous cell carcinomas of the esophagus.[8] These observations suggested that p16 mutations might be involved in the genesis of a wide variety of tumors, as previously described for p53.[9] To clarify the significance of p16 mutations in gastric tumorigenesis, we examined mutations of this gene as well as loss of heterozygosity (LOH) of 9p in gastric adenomas and adenocarcinomas.

Tumorous and normal gastric mucosal tissues were obtained endoscopically from 13 patients with gastric adenoma and surgically from 45 patients with gastric adenocarcinoma, consisting of 31 differentiated and 14 undifferentiated histologic types; these samples were frozen at −80°C. Genomic DNAs were extracted using a phenol-chloroform procedure. Mutations in exons 1 and 2 of p16 were analyzed by the use of polymerase chain reaction (PCR) single-strand conformation polymorphism (SSCP). One hundred nanograms of extracted DNA was amplified by 35 cycles of PCR using a thermal cycler (PJ 2000, Perkin Elmer Cetus Corp., Norwalk, CT) in a buffer (50 m*M* KCl, 0.01% gelatin and 10 m*M* Tris buffer at pH 8.3) containing 20 pmol of each primer, 1 m*M* $MgCl_2$, 0.2 m*M* of each deoxynucleotide triphosphate, 0.5 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus Corp.), 5% dimethyl sulfoxide and 0.5 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 10 Ci/ml). Each PCR cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. PCR primers used for the analysis of p16 mutations were as follows: exon 1, 5′-GAAGAAAGAGGAGGGGCT-G-3′ and 5′-GCGCTACCTGATTCCAATTC-3′[6]; exon 2, 5′-CTGACCATTCTGTTCTCTCTGG-3′ and 5′-C-ATGGTTACTGCCTCTGGTGC-3′. The sense primer sequence for exon 2 is located in intron 1 near the exon-intron boundary, and the anti-sense primer sequence is located at codons 128–135. Over 90% of the coding regions can be analyzed using these primers. LOH on 9p was examined using the microsatellite marker (*IFNA*) on 9p22. Sequences of primers used for the analysis of 9p-LOH were 5′-TGCGCGTTAAGTTAATTGGTT-3′ and 5′-GTAAGGTGGAAACCCCCACT-3′.[10] PCR was performed as described above except that dimethyl sulfoxide was not added. Five microliters of each PCR product was diluted 10-fold with gel-loading buffer (98% deionized formamide, 10 m*M* EDTA pH 8.0, 0.025% xylene cyanol, 0.025% bromophenol blue) and heated at

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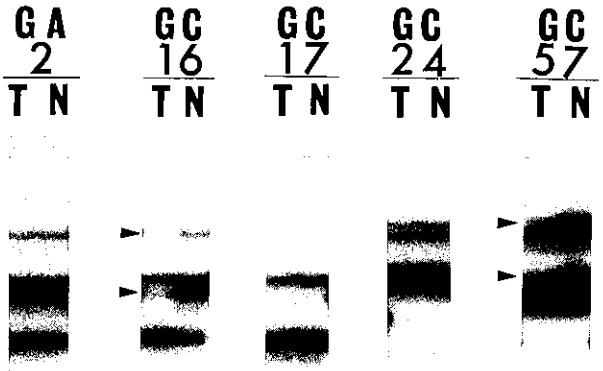

Fig. 1. Analysis of 9p-LOH at the *IFNA* locus. Examples of the analysis are shown and loss of an allele is indicated by arrowheads. LOH was detected in cases GC 16 and GC 57. GA 2 and GC 24 retained heterozygosity, and the analysis was non-informative for GC 17. GA, gastric adenoma; GC, gastric adenocarcinoma; T, tumor; N, normal tissue.

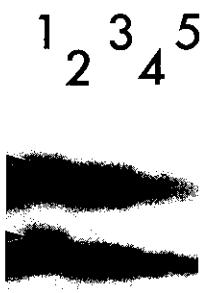

Fig. 2. SSCP analysis of exon 2 of p16. Lane 1, GA 2; lane 2, GC 16; lane 3, GC 17; lane 4, GC 24; and lane 5, GC 57. Mobility shifts in lane 1 (GA 2) are indicated by arrowheads.

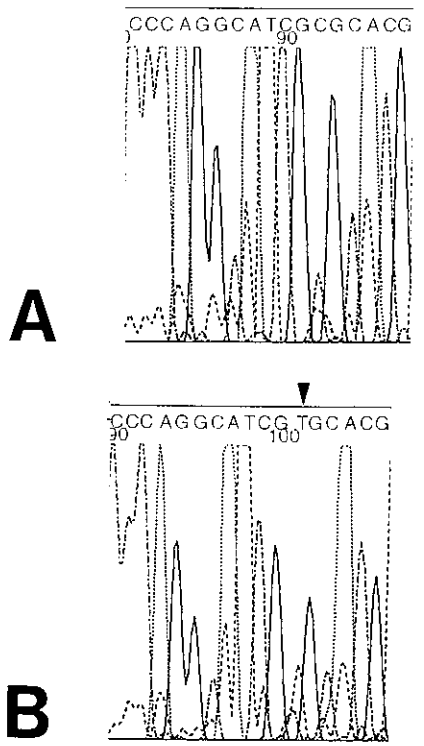

Fig. 3. Sequence histograms of exon 2 of p16 using the antisense primer. (A) Wild-type sequence obtained from the normal SSCP band. (B) Sequence histogram obtained from the mobility-shifted SSCP band (GA 2). A nucleotide change (arrowhead) is present at the second position of codon 99 in this gastric adenoma sample.

94°C for 2 min. Samples were electrophoresed on a 6% neutral polyacrylamide gel at 40 W for 3–4 h for SSCP analysis and on a 6% polyacrylamide gel containing 7 *M* urea at 60 W for 2 h to assess LOH. The gel was dried and exposed on X-ray film at −80°C for 6–12 h. LOH was defined as loss of band(s) corresponding to an allele in tumor DNA. Sequence analysis was performed in the cases with LOH on 9p (*IFNA* locus) based on mobility shift in SSCP using the same primers as used in PCR with a terminator cycle sequencing kit (Taq DyeDeoxy, Applied Biosystems, Foster City, CA) and an automated DNA sequencer (Model 373A, Applied Biosystems).

In the present study, LOH on 9p (*IFNA* locus) was detected in 22% (5/23 informative cases) of differentiated adenocarcinomas, 10% (1/10) of undifferentiated carcinomas and none (0/6) of the adenomas (Fig. 1). Mutations of p16 were not detected in any of the 45 adenocarcinomas or 13 adenomas, although a mobility shift in one gastric adenoma was found by SSCP (Fig. 2). Sequence analysis of the mobility shift band revealed a nucleotide change in the second position of codon 99 (CGC to CAC) in exon 2 (Fig. 3). However, the same mobility shift was detected by SSCP in DNA extracted from normal gastric mucosa of the same patient, and sequence analyses confirmed the same nucleotide change. Therefore, the nucleotide change was considered to be a polymorphism. Since it was possible that we had underestimated the presence of p16 mutations by SSCP, sequence analyses of the six gastric adenocarcinoma cases with 9p-LOH were performed. All these cases revealed wild-type p16 sequences. It remains possible that some mutations were present in the regions not analyzed, although over 90% of coding regions were examined in this study.

Spruck *et al.*[11] reported that although p16 mutations were frequently found in bladder tumor cell lines, the

incidence in primary bladder tumors was three-fold lower. Bonetta hypothesized that p16 may confer a long-term growth advantage in cultured tumor cells.[12] More recently, Cairns *et al.* reported that p16 mutations were detected in only 3% (2/75) of primary tumors of the bladder, head and neck, lung, kidney and brain, all of which showed chromosome 9 loss.[13] Therefore, they suggested the possible existence of another tumor suppressor gene at 9p21-22.[13] Our results also suggest the presence of another tumor suppressor gene on 9p near the *IFNA* locus which may be involved in the development and/or progression of differentiated gastric adenocarcinomas. Our data indicate that p16 mutations probably do not contribute to the genesis of gastric carcinoma, but may play a crucial role in specific tumors such as malignant melanoma and squamous cell carcinoma of the esophagus.

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