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Intragenic Homozygous Deletions of *MTS1* Gene in Gastric Cancer in Taiwan

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The multiple tumor suppressor 1 (*MTS1*) and 2 (*MTS2*) genes, located on chromosome 9p21, have been reported to be deleted or mutated in many malignant cell lines and in a high percentage of some primary carcinomas. To determine whether these genes are altered, and if so, what is the nature of the alterations, in human gastric adenocarcinoma, we investigated their frequency of mutation by Southern blotting, polymerase chain reaction (PCR) and direct sequencing in 55 patients. Furthermore, loss of heterozygosity (LOH) of chromosome 9p21 at the IFNA locus and D9S171 was assessed. Homozygous deletions of exon 1 of the *MTS1* gene were identified in 5 of 55 (9.1%) primary tumors. No deletion of *MTS2* gene was noted. LOH was observed in 7 (14.3%) of 49 informative cases (5 cases at IFNA locus, 2 cases at D9S171 and one case with combined LOH at D9S171 and homozygous deletion at exon 1 of *MTS1*). Direct sequencing of PCR products of the *MTS1* and *MTS2* gene did not reveal any point mutation in these 55 patients. These data indicate that alterations of the *MTS1* and *MTS2* genes are infrequently encountered. Additional studies of LOH with more microsatellite markers near 9p21 are mandatory to elucidate whether another tumor suppressor gene exists in the vicinity of *MTS1* in primary gastric adenocarcinoma.

Key words: Gastric adenocarcinoma — *MTS1* gene — *MTS2* gene — Microsatellite markers — Loss of heterozygosity

Multiple genetic alterations occur during the transformation of normal cells into cancer cells. The evolution may be facilitated by derangements of the cell cycle machinery. Accumulating evidence has shown that genes encoding components of cell cycle checkpoints, when mutated, increase genetic instability and lead to uncontrolled cell growth of a tumor.[1] Among cloned cell-cycle control genes, two highly related members of the cyclin-dependent kinase inhibitor family, termed *MTS1* and *MTS2*, have aroused a great deal of interest because of their potential role in tumorigenesis.[2–8] However, there is a discrepancy of mutational frequency between cell lines and primary tumors.[9–15] Some tumors such as malignant glioma, primary esophageal, pancreatic, and NSCs have been reported to exhibit frequent mutations of the *MTS1* and *MTS2* genes,[9–13] while there is a lower rate of mutations in colorectal, urinary bladder, head and neck, and renal cell carcinoma.[14, 15] Therefore, it remains to be elucidated whether alterations of these genes is tissue-specific or constitutes a general and significant phenomenon in tumorigenesis, as in the case of p53.[16, 17] Furthermore, for GAC, although the molecular genetics with respect to p53, APC, and microsatellite instability has been extensively characterized,[18] reports concerning alterations of the *MTS1* genes are scanty, and we know of no studies on *MTS2* gene.[19, 20] To clarify further the role of *MTS1* and *MTS2* mutations in gastric carcinogenesis, we examined their alterations and allelic loss of chromosome 9p21 in 55 Taiwanese with GAC.

MATERIALS AND METHODS

Tissues and DNA preparation Paired gastric tissues from tumorous and nontumorous sites were obtained during surgery from 55 patients with GAC during the period from January, 1992 to June, 1993 at the National Taiwan University Hospital. To avoid contamination by inflammatory and stromal cells, cryosections of 5–7 μm were prepared for each tumorous tissue and sections containing predominantly neoplastic cells were used for the extraction of genomic DNA by a conventional procedure.[21] DNA from a nontumorous site of the same stomach was also extracted to serve as a control. Among these 55 patients, 14 had early GAC and 41 advanced GAC. Their histologic subtypes were classified as 28 diffuse and 27 intestinal GAC by the same pathologist according to Lauren's classification.[22]

PCR and Southern blotting analysis for *MTS1* and *MTS2* gene mutations Exons 1 and 2 of *MTS1* and *MTS2* genes were amplified by PCR. The primers used for PCR amplification were designed with additional biotinylation at the 5′ end based upon the reported *MTS1* and *MTS2*

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Abbreviations: *MTS1*, multiple tumor suppressor 1; *MTS2*, multiple tumor suppressor 2; GAC, gastric adenocarcinoma; PCR, polymerase chain reaction; LOH, loss of heterozygosity. NSC, non-small cell lung cancer.

Table I. Primer Sequences for p16 and p15 Analyses[a)]

Name	Sequences	Use
p16-1F	5′-GAAGAAAGAGGAGGGGCTG-3′	amplify p16, exon 1
p16-1R	5′-GCGCTACCTGATTCCAATTC-3′	amplify p16, exon 1
p16-2F	5′-GGAAATTGGAAACTGGAAGC-3′	amplify p16, exon 2
p16-2R	5′-GGAAGCTCTCAGCGTACAAA-3′	amplify p16, exon 2
p16-2′F	5′-TCTGAGCTTTGGAAGCTCTCAG-3′	amplify p16, exon 2
p16-2′R	5′-CACAAGCTTCCTTTCCGTCA-3′	amplify p16, exon 2
p16-S1	5′-CGGAGAGGGGGAGAGCAG-3′	sequence p16, exon 1
p16-S2	5′-AGGGGGCTCTACACAAGCTT-3′	sequence p16, exon 1
p16-S2′	5′-TCTCAGATCATCAGTCCT-3′	sequence p16, exon 2
p15-1F	5′-TAATGAAGCTGAGCCCAGGT-3′	amplify p15, exon 1
p15-1R	5′-AATGCACACCTCGCCAACG-3′	amplify p15, exon 1
p15-2F	5′-CTTTAAATGGCTCCACCTGC-3′	amplify p15, exon 2
p15-2R	5′-CGTTGGCAGCCTTCATCG-3′	amplify p15, exon 2
p15-S1	5′-GAAAGAAGGGAAGAGTGTCG-3′	sequence p15, exon 1
p15-S2	5′-CAAGTCCACGGGCAGACG-3′	sequence p15, exon 2
p16-S2′	5′-TCATCGAATTAGGTGGGTGG-3′	sequence p15, exon 2

a) Based on published sequences in references 3 and 6.

sequences (Table I). The PCR reaction was performed in a 50 μl volume containing 200 ng of genomic DNA, 5 μl of 10×PCR buffer, 1 μl of dNTP's (2.5 m*M* each of dATP, dCTP, dGTP, and dTTP), 1.5 μl of primers (20 μM), 0.5 μl of Taq DNA polymerase (5 U/μl), and 5% DMSO. The temperature protocol included 94°C denaturation for 5 min, 94°C (30 s), 62°C (50 s), and 72°C (50 s) for 10 cycles; 94°C (30 s), 60°C (50 s), and 72°C (50 s) for 15 cycles; and 94°C (30 s), 56°C (15 s), and 72°C (30 s) for 20 cycles with a final extension at 74°C for 10 min. All PCR products were verified on ethidium bromide-stained 2% agarose gel electrophoresis with a ϕ ×174 DNA/*Hae* III fragment as a size marker. Southern blotting was performed for further confirmation of homozygous deletions of the *MTS1* and *MTS2* gene. Ten microgram of DNA was digested with *Eco*R I restriction enzyme, separated by electrophoresis in 0.8% agarose gel and transferred to a nylon membrane. PCR-derived probes of exons 1 and 2 of *MTS1* and *MTS2* genes were used individually for hybridization.

Direct DNA sequencing for *MTS1* and *MTS2* genes The PCR products amplified by biotinylated primers of *MTS1* or *MTS2* were immobilized onto a streptavidin-coated solid support (Dynabeads M-280 streptavidin; Dynal AS, Oslo, Norway). A clean single-stranded DNA template immobilized on the solid support was obtained for cycle sequencing by strand-specific elution of the non-biotinylated strand using 0.1 *M* NaOH. DNA sequencing of exon 1 and exon 2 was performed by an Applied Biosystems 373A sequencer (Foster City, CA) using a dye-terminator method with the primers listed in Table I.

LOH analysis Two markers (IFNA and D9S171) near 9p21 were used. PCR was performed in a 25 μl reaction volume containing 5 μM each primer, 0.125 m*M* dATP, 1.25 m*M* each of dGTP, dCTP, and dTTP, 3 μCi α-^{35}S of dATP, 25 ng of DNA, and 0.75 units of Taq DNA

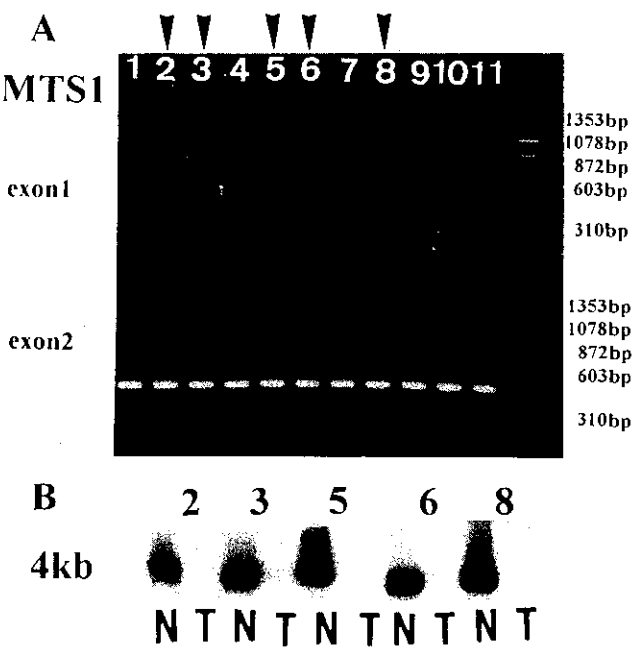

Fig. 1. Homozygous deletions of the *MTS1* gene in gastric adenocarcinoma demonstrated by PCR product electrophoresis (A) and Southern blotting (B). Five cancers (cases 2, 3, 5, 6, 8) exhibit homozygous deletion of exon 1 (arrowheads), but not exon 2. The right-most lane shows ϕ×174/*Hae* III marker. N, normal tissue; T, tumorous tissue.

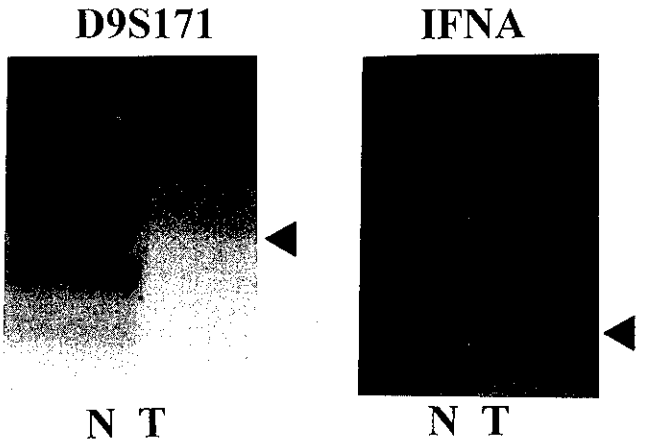

Fig. 2. Representative examples of loss of heterozygosity (arrowheads) at D9S171 and IFNA locus. N, normal tissue; T, tumorous tissue.

polymerase (Boehringer Mannheim, Mannheim, Germany). The reaction conditions consisted of 30 s at 94°C, 75 s at 55°C, and 15 s at 72°C for 27 cycles followed by a final extension for 5 min at 72°C. The PCR products were electrophoresed on 7% polyacrylamide sequencing gels. After electrophoresis, gels were dried at 80°C and exposed to X-ray film from 24 to 72 h. The band pattern was compared between tumorous and nontumorous tissues for each patient.[21]

RESULTS

No point mutation was detected for *MTS1* or *MTS2* gene in these 55 patients. However, five of 55 (9.1%) AGC were noted to have homozygous deletions of exon 1 of the *MTS1* gene, which was demonstrated by PCR and Southern blotting (Fig. 1). Among these 5 cases with deletions on exon 1, one was early GAC and the other four were advanced GAC. Four belonged to the intestinal type and one to the diffuse type of GAC. LOH on 9p21 was detected in 7 (14.3%) of 49 informative cases including 2 at D9S171 and 5 at the IFNA locus. Representative examples of LOH are shown in Fig. 2. Among these 7 cases, 6 were advanced cancer and 5 were of intestinal type. One patient with intestinal advanced cancer has LOH at D9S171 and homozygous deletion of *MTS1*.

DISCUSSION

In the present study, homozygous deletions of *MTS1* were found in 9.1% of GAC. This frequency is much lower than that of primary glioma, esophageal, pancreatic and NSCs.[9–13] Because the use of *in vivo* cancer specimens can lead to underestimation of the occurrence of homozygous deletions due to contaminating inflammatory and stromal cells,[23] we employed cryosections and Southern blotting to minimize technical error. Two recent reports from Japan also indicated that *MTS1* gene alterations rarely occur in GAC.[19,20] Taking these data together, we conclude that *MTS1* mutations play a limited role in gastric tumorigenesis and that the frequency of *MTS1* mutations indeed varies considerably with different tumor types.

Since a neighboring gene *MTS2*, with biochemical properties similar to those of *MTS1*, has recently been shown to be deleted together with the *MTS1* gene in glioblastoma multiforme and NSCs,[6,7] it was speculated that homozygous deletions, rather than intragenic mutations, may be more efficient for simultaneous inactivation of both genes. For GAC, no extensive search for *MTS2* alterations has been documented. Since we found neither homozygous deletion nor point mutations of *MTS2* gene, it appears that *MTS2* gene alterations play no role in gastric tumorigenesis. Recently, Cairns *et al.* found that a 170 kb region that includes *MTS1* but excludes *MTS2* is responsible for the homozygous deletions of 9p21 encountered in various primary tumors.[23]

Nevertheless, the possibility of other coexistent target genes in addition to *MTS1* on 9p21 cannot be completely excluded.[17] Frequent loss of heterozygosity on chromosome 9 with low incidence of mutations of *MTS1* and *MTS2* gene has been documented in malignant mesothelioma and glioma.[24,25] We noted a certain portion (14.3%) of GAC with LOH at D9S171 and the IFNA locus, but without point mutations in the *MTS1* gene. Moreover, 5 of 7 cases with LOH were intestinal-type advanced GAC. This result is consistent with a recent Japanese report which suggested that another tumor suppressor gene on 9p21 near the IFNA locus may contribute to the progression of differentiated adenocarcinoma of the stomach.[19] Additional studies to search for any LOH at 9p with more microsatellite markers and to determine the frequency of *MTS1* and *MTS2* alterations in other primary tumors are needed to elucidate the roles, if any, of these genes in tumorigenesis.

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