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Frequent Microsatellite Instabilities and Analyses of the Related Genes in Familial Gastric Cancers

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Microsatellite instability or replication error seems to be related to defective DNA mismatch repair genes, such as *hMSH2, hMLH1, hPMS1* and *hPMS2*, which have been identified as causative genes of hereditary nonpolyposis colorectal cancers (HNPCC). Recently, it was reported that mutations at the simple repeated sequences in the transforming growth factor-β type II receptor (*TGF-β RII*) gene occurred in replication error-positive colorectal cancers. To determine genetic alterations in familial gastric cancers (FGC), we examined replication error using eight microsatellite DNA markers, and screened mutations in the *hMSH2, hMLH1* and *TGF-β RII* genes in six cases from four FGC kindreds. Moreover, *hMTH1*, a human homolog of the bacterial *mutT* gene, was also screened. Four of six (67%) cancers showed the replication error-positive phenotype, indicating that microsatellite instability is highly associated with not only HNPCC, but also FGC. No germline mutation was found in the whole coding sequences of *hMSH2* and *hMTH1*, or in the conservative regions of *hMLH1* in any patient, while one cancer DNA showed a somatic mutation at codon 682 (threonine to alanine) in *hMSH2*. No alteration was found at the small repeated sequences in *TGF-β RII* in FGC tumor DNA. These results indicate that the carcinogenetic process of FGC may be different from that of HNPCC.

Key words: Familial gastric cancer — Microsatellite instability — DNA mismatch repair gene — Replication error

Several hereditary tumors, such as familial adenomatous polyposis and retinoblastoma, have been reported, and the genes responsible for these tumors, *APC* and *RB-1*, respectively, have been cloned.[1–3] However, there have been very few clinical and genetic studies on familial predisposition to gastric cancers. Since gastric cancers are very frequent in Japan, we were interested in surveying Japanese families with a high incidence of gastric cancers.

Microsatellite instability or replication error (RER) at simple repeated sequences has been reported in hereditary nonpolyposis colorectal cancers (HNPCC)[4,5] and several types of sporadic cancers, including gastric cancers.[6–8] Since 18–39% of sporadic gastric cancers showed RER,[6–8] RER may play an important role in the development of gastric cancers. On the other hand, in the two reports on microsatellite instability in gastric cancers with a family history,[9,10] the incidences of RER were quite different, and thus it is not clear whether RER is also related to familial gastric cancers (FGC).

Recently, four causative genes in HNPCC, *hMSH-2*,[11,12] *hMLH1*,[13,14] *hPMS1* and *hPMS2*,[15] were identified as homologs of bacterial DNA mismatch repair genes. RER may be caused by these defective mismatch repair genes and is likely to play an important role in colorectal carcinogenesis. For example, a defect in the small repeat sequences of the transforming growth factor-β type II receptor (*TGF-β RII*) gene seems to be highly associated with RER(+) sporadic colorectal cancers and HNPCC.[16] We also reported that 71% of RER(+) HNPCC cancers revealed mutations at the $(A)_{10}$ simple repeated sequence in *TGF-β RII*, and suggested that mutations of *TGF-β RII* were highly associated with HNPCC carcinogenesis.[17]

To clarify genetic alterations in FGC, we analyzed RER using microsatellite markers. Since several RER (+) gastric cancers were found in our FGC cases, we further searched for mutations in the mismatch repair genes, *hMSH2* and *hMLH1*. Moreover, to determine whether or not the mechanism of carcinogenesis in FGC is similar to that in RER(+) colorectal cancers, we also analyzed alterations in small repeat sequences of *TGF-β RII*. Additionally, *hMTH1*, a human homolog of *Escherichia coli mutT*, a defect of which would not induce the RER(+) phenotype,[18–20] was examined for mutations, since the mechanism of carcinogenesis in RER(−) HNPCC as well as FGC has not yet been elucidated.

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MATERIALS AND METHODS

Identification of FGC Identification of patients with a family history of gastric cancers was carried out with the following criteria. 1) At least three relatives should have gastric cancer; and one of them should be a first degree relative to the other two. Other hereditary tumors, such as cancer family syndrome (Lynch II) of HNPCC,[21] should be excluded. 2) At least two successive generations should be affected. 3) In one of the relatives gastric cancer should have been diagnosed at under 50 years of age. According to these criteria, four families were classified as FGC in this study.

Subjects Gastric cancers and corresponding normal tissues were obtained surgically from six patients of four FGC kindreds. Lymphocytes were purified from fresh peripheral blood from the FGC patients, and were infected with Epstein-Barr virus and established as cell lines.

Analyses of microsatellite instability and loss of heterozygosity (LOH) Genomic DNA was extracted from frozen or paraffin-embedded tissues as described previously.[22, 23] We examined DNA for microsatellite instability and LOH using loci containing the $(CA)_n$ repeat (D2S119, D2S123, CA21, D3S1029, D3S1611, D10S197, D13S175)[4, 24] or a locus containing the $(A)_n$ repeat (BAT26).[17, 25] Polymerase chain reaction (PCR) was performed in 50 μl reaction mixtures containing 2 μl of template DNA, 10 pmol of each oligonucleotide primer pair (one end-labeled with [γ-^{32}P] ATP), 5 U of *Taq* DNA polymerase, 5 μl of 10× buffer (Wako Co., Ltd., Osaka), and 1 μl of 1.25 mmol dNTP (Pharmacia, Uppsala, Sweden).

Each PCR involved 30 cycles of denaturation for 1 min at 94°C, annealing for 2 min at 53–60°C, and extension for 1 min at 72°C, followed by final extension for 10 min at 72°C. The PCR products were denatured in 95% formamide for 2 min at 94°C and then electrophoresed on denaturing 6% polyacrylamide sequencing gels. After electrophoresis, the gels were exposed to X-ray film for 2–48 h. When RER was found in two or more loci in a tumor, this tumor was determined to have the RER(+) phenotype. On the other hand, tumors without any marker or with only one positive marker were regarded as RER(−).

Analyses of *hMSH2*, *hMLH1*, *hMTH1* and *TGF-β RII* for mutations The primers used for genomic PCR were synthesized for all exons of *hMSH2*,[26, 27] and exons 2, 9, 14–16 and 19 of *hMLH1*,[28, 29] which are among the most conservative regions. Moreover, exons 2, 3 and 4 of *hMTH1*,[20] which cover all the coding exons, were also amplified. We also synthesized primers for codons 1–290, 253–397, 382–784 and 684–934 of *hMSH2*, codons 680–756 of *hMLH1*, and the whole coding region of *hMTH1*, for cDNA templates generated from mRNA of lymphoblastoid cells. Reverse transcription (RT)-PCR was carried out as described previously.[27] The primers used for genomic *TGF-β RII* were synthesized for nucleotide positions 677–766 and 1886–2009 containing small repeat sequences $(A)_{10}$ and $(GT)_3$, respectively.[17] The sequences of these primers used for amplification and sequencing of *hMSH2*, *hMLH1*, *hMTH1* and *TGF-β RII* are available from the authors upon request.

PCR-single strand conformation polymorphism (SSCP) analysis was performed as described previously.[30] Briefly, PCR was carried out for 35 cycles. The PCR products were denatured and then electrophoresed on 10–12.5% non-denaturing polyacrylamide gels containing 10% glycerol in Tris-glycine buffer (25 m*M* Tris-HCl, 200 m*M* glycine, pH 8.3). After electrophoresis, the gels were stained with silver (Dai-ichi Co., Ltd., Tokyo).

Sequencing When abnormal patterns were observed on SSCP analysis, the PCR products were sequenced directly with a cycle sequencing kit (Takara, Kyoto). For exon 13 of the *hMSH2* gene, the PCR product was cloned into the pT7Blue(R) T-vector (Novagen, Madison, WI). Several subclones were sequenced with a Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH).

RESULTS

Six patients from four families satisfied the criteria of FGC. The clinical and pathological findings in these cases are summarized in Table I, and a representative pedigree is shown in Fig. 1.

Alterations of the electrophoretic pattern of microsatellite in tumor DNA were analyzed in comparison with that in normal DNA. Four of the six (67%) tumors showed the RER(+) phenotype at two or more loci, while the other one (tumor G14) showed RER in only one marker (Table I). These RER(+) tumors showed both deletions and insertions in the microsatellite repeats, similar to those described for RER(+) HNPCC tumors. Representative alteration patterns between normal and cancer DNA with D2S123, D3S1029 and D13S175 are shown in Fig. 2. Only one tumor (G6) showed LOH in the D13S175 region, but no LOH was found as to D2S119, D2S123 or CA21 for *hMSH2*, or D3S1029 or D3S1611 for *hMLH1*, respectively, in the six FGC tumors.

No germline mutation was detectable in any exon of *hMSH2* using genomic DNA of the six FGC patients. A transition of CTT (leucine) to TTT (phenylalanine) at codon 390 of *hMSH2* was found in one case. However, this alteration was also detected in 2 of 15 healthy individuals, which suggested that it might be a polymorphic change. Then we screened for germline mutation in codons 680–756, including exons 17–19, of the *hMLH1*

Table I. Incidence of Microsatellite Instability in Tumors from FGC

Family	Patient	Age	Sex	Histology[a]	Invasion[b]	D2S119	CA21	D2S123	D3S1029	D3S1611	D10S197	D13S175	BAT26
FGC1	G1	71	M	tub1	sm	−	+	+	+	−	−	+	−
	G6	57	M	por	ss	−	−	−	−	−	−	LOH	−
FGC2	G2	63	M	tub1	sm	−	−	+	+	+	−	+	+
	G3	35	M	sig	pm	−	−	+	−	−	+	−	−
FGC3	G15	58	F	tub2	pm	+	ND[c]	+	+	+	+	−	−
FGC4	G14	17	M	tub2	s	−	−	−	−	−	−	+	−

a) The histological type of tumors was classified according to the General Rules for the Gastric Cancer Study in Surgery and Pathology.[36] tub1: well differentiated adenocarcinoma, tub2: moderately differentiated adenocarcinoma, por: poorly differentiated adenocarcinoma, sig: signet-ring cell carcinoma.
b) The depth of invasion of tumors was also classified according to the General Rules. sm: carcinoma limited to within the mucosa and submucosa, and not reaching the proper muscle, pm: carcinoma limited to the proper muscle, ss: carcinoma extending from the mucosa beyond the proper muscle but not exposed to the serosal surface, s: carcinoma exposed to the serosal surface.
c) ND: not determined.

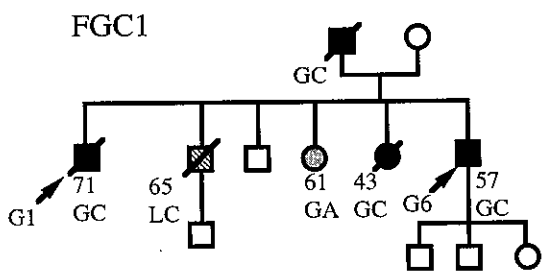

Fig. 1. Pedigree of FGC1. The individuals (G1 and G6) from whom DNA was available are indicated by arrows. The age at diagnosis is shown below each member. Open symbols, no cancer occurred; GC, gastric cancer; GA, gastric adenoma; LC, liver cancer.

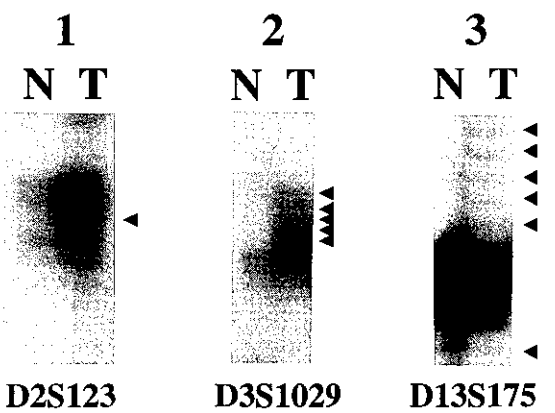

Fig. 2. Representative results for the RER phenotype using three microsatellite markers (D2S123, D3S1029 and D13S175) in FGC. Alterations of the electrophoretic pattern of the $(CA)_n$ repeat in cancer DNA (T) were analyzed in comparison with that in corresponding normal DNA (N). Mobility shifts indicating RER are shown by arrowheads. Lanes 1 and 3, patient G2; lane 2, patient G15.

cDNA and also for germline and/or somatic mutation in exons 2, 9, 14–16 and 19 of *hMLH1* in the six FGC patients. However, no mutation was found in these regions on PCR-SSCP.

As for exon 13 of *hMSH2*, tumor DNA from patient G3 showed an abnormal SSCP pattern in comparison with his normal DNA (Fig. 3A), indicating a somatic mutation. The PCR products of exon 13 of *hMSH2* in normal and tumor DNA from this patient were subcloned and then sequenced. The sequence of cancer G3 contained GCT (alanine) at codon 682 in one allele, although the sequence of the normal DNA contained ACT (threonine) in both alleles (Fig. 3B). Since this nucleotide change created a new restriction site for *Alu* I, the PCR products from the six FGC tumors were digested with *Alu* I and then electrophoresed on a 15% polyacrylamide gel. Only tumor G3 was digested with *Alu* I at one allele (183+47 bp), while an undigested band (230 bp) reflecting a wild-type allele was detected for normal DNA from patient G3 and other gastric cancers (Fig. 3C).

None of the six FGC tumors showed an abnormal band for nucleotide positions 677–766 or 1886–2009 of *TGF-β RII* on PCR–SSCP, whereas RER(+) cases of HNPCC did exhibit abnormal patterns for nucleotide positions 677–766 (data not shown).

In the *hMTH1* study, specific mutations in coding exons of *hMTH1* were not detected in either normal or tumor cells from the six FGC patients on PCR-SSCP analysis. We also examined the whole coding sequences of the *hMTH1* cDNA in cases G1 and G3, but no mutations were found (data not shown).

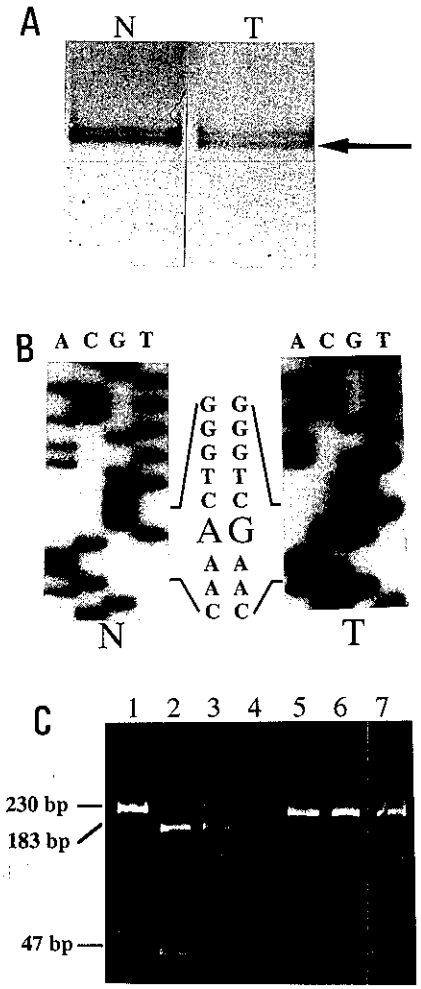

Fig. 3. A) PCR-SSCP analysis of exon 13 of the *hMSH2* gene in patient G3. The PCR products were electrophoresed on a 12.5% non-denaturing polyacrylamide gel containing 10% glycerol and then stained with silver. The mobility shift is indicated by an arrow. N, normal; T, cancer. B) Sequencing analysis of the PCR products from patient G3. The PCR products from normal (N) and cancer DNA (T) were cloned into the pT7Blue(R) T-vector and then sequenced. C) *Alu* I digestion of the PCR products from FGC patients. The PCR products from cancer DNA (patient G3, lane 3) and its clone (lane 2) showed mutant bands (183 and 47 bp), while the PCR products from normal G3 DNA (lane 4), other FGC tumors (lanes 5 and 6), and a healthy individual (lane 7) showed an undigested band (230 bp). Lane 1, undigested PCR product of lane 2.

DISCUSSION

Four FGC kindreds were studied as to genetic alterations. There were no specific clinical and pathological findings in these FGC kindreds. We found the RER(+) phenotype in four of the six (67%) FGC tumors. It was reported that 18–39% of sporadic gastric cancers showed the RER(+) phenotype[6–8] and two cases of gastric cancer-prone families also revealed it.[10] Our results, together with the previous data,[10] indicate that the incidence of RER in FGC is higher than that in sporadic gastric cancers, and that microsatellite instability is highly associated with not only HNPCC, but also FGC. However, there was a different RER pattern between HNPCC and FGC. RER of mononucleotide repeats was highly frequent in HNPCC,[25] while RER of a mononucleotide repeat, BAT26, was observed only in one of the six FGC tumors.

Several HNPCC and RER(+) sporadic colorectal cancers have been reported to show mutations in mismatch repair genes,[11–15, 26–29] so the RER(+) phenotype might be induced by the inactivation of mismatch repair genes.[26–29] It was demonstrated that 80–90% of HNPCC is linked to *hMSH2* or *hMLH1* by a linkage study.[31] We searched for mutations in all the exons of the *hMSH2* genes, and exons 2, 9, 14–19 of the *hMLH1* gene, which include the most conservative regions and mutated codons,[13, 14, 28] through SSCP and sequencing analyses on our FGC patients. No germline mutation was detectable in the six FGC cases, suggesting that mutations in *hMSH2* or *hMLH1* are not likely to be associated with a marked FGC predisposition.

An amino acid change from threonine to alanine at codon 682 in *hMSH2* was found in cancer G3 but not in corresponding normal cells on *Alu* I digestion, suggesting a somatic mutation. The inactivation of both alleles in *hMSH2* or *hMLH1* might be required for carcinogenesis in HNPCC and RER(+) sporadic colorectal cancers.[24, 27] We found a somatic mutation in one allele but not in the other allele in the case of cancer G3. Therefore, we could not define the candidate gene causing RER in this family, even though the G3 cancer showed RER.

LOH was found in only one tumor using D13S175, suggesting that LOH in D13S175 is rare in FGC. It has already been reported that LOH occurred in the *hMSH2*[27] or *hMLH1* gene[24] in several HNPCC tumors. However, the six FGC tumors showed no LOH in D2S119, D2S123 or CA21 for *hMSH2*, or D3S1029 or D3S1611 for *hMLH1*, respectively. These data suggest that LOH of mismatch repair genes may not be associated with FGC carcinogenesis.

In this study, we did not screen mutations in *hPMS1* and *hPMS2*, since germline mutations of these genes are rare in HNPCC.[15] Although *hPMS3* to *hPMS8* have been identified as homologs of yeast *PMS* genes,[32] it is uncertain whether these genes are related to HNPCC or several types of sporadic cancers with microsatellite instability. Since germline mutations in *hMSH2* and

hMLH1 were not detectable in our FGC cases, it is possible that one of the *hPMS* genes or other mismatch repair genes related to genomic instability might be a candidate gene for FGC.

Recently, a new kind of DNA mismatch repair gene, G/T binding protein (*GTBP*), was reported.[33,34] In *GTBP*-defective cells, dinucleotide repeats seem to be stable but mononucleotide repeats do not. On the other hand, both di- and mononucleotide repeats are instable in colorectal cancer cells, which are defective in *hMSH2, hMLH1, hPMS1* or *hPMS2*. When we analyzed one mononucleotide repeat in the six FGC tumors, only one showed a change. This tumor (G2) also exhibited a $(CA)_n$ change. Therefore, none of the six FGC tumors showed the *GTBP*-defective phenotype as to RER. These data suggest that *GTBP* is not related to FGC.

It has been reported that alterations at simple repeat sequences, that is, $(A)_{10}$ and $(GT)_3$, in *TGF-β RII* have been found in RER(+) colorectal cancers and HNPCC, suggesting that *TGF-β RII* is the target gene of RER and thus is highly associated with colorectal carcinogenesis.[16,17,25] Recently, one of three gastric cancers in patients with family history,[10] one case of gastric cancer from HNPCC,[17] and five of seven RER(+) sporadic gastric cancers[35] also exhibited mutation in the $(A)_{10}$ sequence. If the carcinogenesis of the four RER(+) FGC tumors is quite similar to that of cancer family syndrome (Lynch syndrome II[21]) in HNPCC, it is possible that the mutation in the $(A)_{10}$ repeat of *TGF-β RII* also occurs in these tumors. In this study, however, no alterations were detectable at either the $(A)_{10}$ or $(GT)_3$ repeats in the six FGC tumors. These results indicate that alterations at simple repeats in *TGF-β RII* might be rare in FGC. It is not clear why the mutation frequency in *TGF-β RII* is different between RER(+) sporadic gastric cancers and FGC.

We defined the RER(+) phenotype as that when cancer DNA showed genomic alterations in more than one of eight microsatellite markers. Two cancers showed microsatellite alterations in less than two markers, indicating the RER(−) phenotype. Therefore, the genetic features of these RER(−) gastric cancers might be different from that of RER(+) cancers. The bacterial *mutT* homolog, *hMTH1*, has different properties from mismatch repair genes, since the function of the *hMTH1* gene product is related to the metabolism of an altered nucleotide, 8-oxo-dGTP, but not to a replication error.[18,19] To determine whether or not *hMTH1* is responsible for FGC, we screened this gene in detail by PCR-SSCP and direct sequencing. Neither germline nor somatic mutations were detectable in the whole coding region of *hMTH1*, suggesting that *hMTH1* is not associated with FGC.

In summary, FGC tumors revealed a high frequency of microsatellite instability, but there are some differences between FGC and HNPCC. Defective *hMSH2* or *hMLH1*, high frequency of mononucleotide repeat alterations and inactivation of *TGF-β RII*, which occurred in most HNPCC, are not likely to be associated with RER(+) FGC tumorigenesis. These data indicate that FGC may not share the same carcinogenetic process as HNPCC. It is important to collect more FGC cases and to analyze their genetic alterations further.

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