RER phenotype and its associated mutations in familial gastric cancer

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To clarify the genetic background of gastric cancer, we collected 28 familial gastric cancers (FGCs) with reference to the Amsterdam criteria in hereditary non-polyposis colorectal cancer (HNPPC) and investigated the frequency of replication error (RER) at six microsatellite loci and frameshift mutations in its related genes in these tumors. RER was detected in seven (25%) of the 28 gastric cancers. Five (18%) cases showed RER at more than two loci. The apparent increased incidence of RER in FGC was not detected compared with that reported in sporadic gastric cancers previously. Among four cases with RER at more than three loci, frameshift mutations in the (A)8 track of the hMSH3 gene were detected in all the four cases and mutations in the (A)10 track of the transforming growth factor-β type II receptor (TGF-β RII) gene were detected in the three of them. Histologically, three of the four cases were of the intestinal type, and the other one was the diffuse type. No mutation was detected in the (C)8 and (GT)3 region of the hMSH6 and TGF-β RII genes respectively. These results indicate that the acquisition of the RER phenotype equally influences the gastric carcinogenesis of both sporadic and familial cases, and that the majority of FGC is pathogenetically distinct from HNPCC.

Introduction

Epidemiological studies have shown that there is a familial clustering in gastric cancer (1–4). However, it has not been clear that this familial aggregation is either caused by inherited gene alterations or by environmental factors shared by family members. Since gastric cancer is common in hereditary non-polyposis colorectal cancer (HNPPC) (5), familial aggregation of gastric cancers may exist in HNPCC families. Previously we reported two cases of gastric cancers with replication error (RER) phenotype among four familial gastric cancers (FGC) (6), and recently others also reported the presence of RER phenotype in FGC (7). Therefore it is possible that inherited disorders in the mismatch repair system is responsible for FGC as in the case of HNPCC. However, since the number of cases in the previous studies was small and no RER in members of familial cases was also reported (8), we examined for RER phenotype in a large number of familial cases.

Abbreviations: FGC, familial gastric cancer; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; HNPCC, hereditary non-polyposis colorectal cancer; TGF-β RII, transforming growth factor-β type II receptor; RER, replication error.

Recently, a target of RER phenotype was identified in the (A)10 repeat of the transforming growth factor-β type II receptor (TGF-β RII) gene (9–11). Simple repeat sequences in the mutator genes (hMSH3 and hMSH6) are also suggested as being other targets (12). Frameshift mutations in TGF-β RII were detected in most of colorectal cancers with RER phenotype (90%) (11), while hMSH3 (A)8 and hMSH6 (C)8 were mutated in 39% and 30% of colorectal cancers with RER, respectively (12). In the TGF-β RII gene, a mutation in the (GT)3 region was also detected in a colorectal cancer cell line (9,11). Thus, we also examined frameshift mutations in these three RER-associated genes to clarify whether accumulation of mutations in these genes is involved in the development of FGC.

Materials and methods

Sample collection

Considering the high incidence of gastric cancer in Japan, there may be many families that by chance have two or more patients with gastric cancer. Therefore we strictly defined FGC according to the following criteria, with reference to the Amsterdam criteria in HNPCC (13). (i) At least three relatives should have gastric cancer, and one of them should be a first-degree relative of the other two. (ii) At least two successive generations should be affected. (iii) In one of the relatives, gastric cancer should be diagnosed before age 50. Previously we obtained the information that 0.9% of the gastric cancer patients registered in 1962 through 1995 in the National Cancer Center Hospital (Tokyo, Japan) fit to these criteria (unpublished). In this study, 28 families were collected according to these criteria.

Gastric cancers from a total of 28 FGC cases and corresponding normal mucosa were analyzed. Samples, fixed with formalin and embedded in paraffin, were obtained from surgically resected stomach at Sendai Red Cross Hospital (Sendai, Japan), Yamagata Prefectural Central Hospital (Yamagata, Japan), National Defense Medical College Hospital (Tokorozawa, Japan), Matsuhash Memorial Hospital (Osaka, Japan) with the cooperation by the members of the Japanese Research Society for Gastric Cancer (14). Gastric cancers were histologically classified according to the criteria of Japanese Research Society for Gastric Cancer and Lauren’s criteria (15,16). The tumors were microscopic-al dissected with minimum incorporation of normal tissues, and genomic DNA was extracted as described previously with some modifications (17,18).

Microsatellite analysis

Six microsatellite loci, D2S136 (chromosome 2p) (19), D3S1067 (3p) (20), DSS421 (5q) (19), D6S87 (6q) (21), DBS167 (8q) (19) and TP53 (17p) (22) were examined for RER. Polymerase chain reaction (PCR) was carried out in 15 µl of reaction mixtures containing 1 µl of genomic DNA, 10 nM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% gelatin, 1.5–3.0 mM MgCl2, 10 pmol of each primer, 200 µM of each dNTP, 0.2 µl of α-32P-labeled dCTP (3000 Ci/mmol; 10 Ci/mmol) and 1 unit of Taq DNA polymerase (Pharmacia, Piscataway, CA). DNA was amplified with 35 cycles of denaturation (40 s at 94°C), annealing (40 s at 56°C) and extension (90 s at 72°C) followed by a final elongation (10 min at 72°C). The amplified products were then denatured in 95% formamide and electrophoresed on a 5% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel was fixed on a 3 MM paper (Whatman, England) and autoradiographed (Kodak XAR-5 film, Rochester, NY) for 24 h. The identification of the abnormal patterns in tumors compared with those in normal tissues was performed blindly. We categorized a tumor with expansion and/or contraction of CA repeats at one or more microsatellite loci as having the RER phenotype. All cases showing the RER phenotype were verified by duplicate experiments. In preliminary experiments, expansion or contraction of bands due to alterations of the numbers of CA repeats was confirmed by DNA sequencing (data not shown).

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Mutation analysis of the hMSH3, hMSH6 and TGF-β RI genes

All tumors and corresponding normal tissues were screened for frameshift mutations of the regions including the following small repetitive sequences: (A)₁₀ in the coding region of the hMSH3 gene (codons 381–383), (C)₈ in the coding region of the hMSH6 gene (codons 1116–1118), and (A)₁₀ in nucleotides 709–718 and (GT)₃ in nucleotides 1872–1877 and 1931–1936 of the TGF-β RI gene. The primer sequences were 5'-AGATGCTGATCCCTAATCAAGC-3' and 5'-ACTCCACAAATGCAATTTAAAATG-3' for hMSH3, 5'-GGGTGATGTGCTTATGTCTC-3' and 5'-CCTATGCAAGATCCGCTG-3' for hMSH6, 5'-AGATGCTGCTTCTTTGCAATGCT-3' and 5'-TTCAGCTATCAGT-3' for (A)₁₀ in TGF-β RI, and 5'-CCCAGCTACAGGCGATCCAGA-3' and 5'-GACATGCTTCCCGGCAGA-3' for two (GT)₃ in TGF-β RI.

PCR-single strand conformation polymorphism (SSCP) analysis was performed as follows. PCR products were diluted 10-fold with formamide dye solution. After the samples were heated at 80°C for 5 min, diluted samples were electrophoresed on a 5% polyacrylamide gel with/without 5% glycerol with cooling by a fan in a cold room. PCR products were also separated by electrophoresis on a 5% polyacrylamide/7 M urea gel. Assays of cases showing shifted bands were repeated.

DNA sequencing

The aberrant DNA fragments were extracted from a gel corresponding to the position of shifted bands with distilled water. DNA was reamplified with the same set of primers as the one used for the first amplification. Reamplified DNA fragments were subcloned into the pCR II vector (Invitrogen, San Diego, CA). Several subclones were sequenced in both directions using DNA sequencing system (Promega, Madison, WI) according to the manufacturer's instructions.

Results

Histopathologically, 28 FGC cases consisted of 14 intestinal types (corresponding to differentiated type in Japanese classification system) and 14 diffuse types (undifferentiated-type) according to the Lauren's criteria, and 11 were early stage (intrasubmucosal) and 17 were advanced stage (extending to subserosa) tumors. All tumors and corresponding normal tissues were screened for frameshift alterations and mutations in small repetitive sequences of the hMSH3 and hMSH6 genes. The cases with RER phenotype and/or mutations of these genes are summarized in Table I.

Seven of the 28 cases (25%) showed RER at one or more loci. Five cases (18%) showed RER at two or more loci (Figure 1A). SSCP variants were detected in the PCR products containing the (A)₁₀ repeat of the TGF-β RI gene and the (A)₈ repeat of the hMSH3 gene in three and four cases respectively, whereas they were not in the (GT)₃ region of the TGF-β RI gene and the (C)₈ repeat of the hMSH6 gene (Figure 1B and C). Sequence analysis of DNA fragments revealed one base deletion in the sequence of the (A)₁₀ (data not shown) and (A)₈ repeats (Figure 1D). These deletions were predicted to produce truncated proteins through frameshift mutations.

Histologically, early stage cancer was likely to have RER at one or more loci (three out of 11 cases, 27%), but only one case had RER at the two or more loci. Four cases with RER at more than three loci were all in advanced stages, and three of them were the intestinal type according to the Lauren's criteria. All the cases, in which frameshift mutations were detected in the TGF-β RII and/or hMSH3 gene, showed RER at more than three loci. There were no colorectal cancer cases in their families.

Discussion

The presence of microsatellite instability has been reported in several cases of FGC, although the criteria of FGC and the incidence of RER was not consistent among the reports (6–8,18) (Table II). Thus, pathogenetic significance of the RER phenotype in FGC is still unclear. We detected the RER phenotype in seven of 28 cases (25%) at more than one locus and in five cases (18%) at more than two loci. The incidence of RER in FGC was not significantly different from that in sporadic cases (Table II) (23–32), although stage-matched comparison is not possible among published reports. This result indicates that the acquisition of RER phenotype does not largely contribute to familial clustering of gastric cancer. The result also indicates that the majority of familial clustering of gastric cancer is pathogenetically different from HNPCC.

Histopathologically, acquisition of RER in the progression of gastric cancer (26) was not significant in our FGC cases. Probably it reflects the relatively frequent occurrence of RER in early stage cancer in our series. Although this observation is consistent with our previous communication (18), further rigid comparison is definitely needed. Three of four cases with more than three RER were of the intestinal type, although the distribution of histological subtypes in our series are not different from the one in general (33). The predominance of the intestinal type in tumors with RER phenotype is consistent with the data by Chung et al. (34), although the reason for this is not fully known.

Frameshift mutations in the (A)₁₀ sequence of the TGF-β RI gene have been frequently detected in HNPCC and sporadic colon cancer with RER phenotype (9,11,35). This mutation was predicted to yield truncated, non-functional proteins, resulting in the loss of growth inhibition by TGF-β. Therefore,

<table>
<thead>
<tr>
<th>Case</th>
<th>No. of RER</th>
<th>Age</th>
<th>Sex</th>
<th>Histology</th>
<th>Stage</th>
<th>Frameshift mutation</th>
<th>Locus with RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>4</td>
<td>67</td>
<td>F</td>
<td>Diffuse</td>
<td>Advanced</td>
<td>–1ᵇ</td>
<td>D2S136, D5S421, D8S167, TP53</td>
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<td>3</td>
<td>3</td>
<td>81</td>
<td>F</td>
<td>Intestinal</td>
<td>Advanced</td>
<td>–1</td>
<td>D2S136, D3S1067, TP53</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>48</td>
<td>F</td>
<td>Intestinal</td>
<td>Advanced</td>
<td>–1</td>
<td>D2S136, D3S1067, D5S421</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>64</td>
<td>F</td>
<td>Intestinal</td>
<td>Advanced</td>
<td>wt</td>
<td>D5S421, D8S167</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>43</td>
<td>F</td>
<td>Diffuse</td>
<td>Early</td>
<td>wt</td>
<td>D5S421</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>46</td>
<td>F</td>
<td>Intestinal</td>
<td>Early</td>
<td>wt</td>
<td>D5S421</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>50</td>
<td>M</td>
<td>Intestinal</td>
<td>Early</td>
<td>wt</td>
<td>D6S857</td>
</tr>
</tbody>
</table>

ᵃ Histological classification according to the Lauren's criteria.
ᵇ –1 and wt indicate one base deletion and wild type, respectively.
Fig. 1. Microsatellite analysis and mutation analysis of the TGF-β RII and hMSH3 genes in FGC. In each case, paired normal (N) and tumor (T) DNA are presented. (A) RER phenotype detected in FGCs that showed mutations in the TGF-β RII and hMSH3 genes. In tumor DNAs, expanded and/or contracted bands are detected at each microsatellite locus. (B) PCR-SSCP analysis for the (A)_10 track of the TGF-β RII gene in these cases. Colon cancer case is a positive control for TGF-β RII mutation (two base deletion). Mobility shifts were detected in tumor DNAs. One base deletion was confirmed by sequencing. (C) PCR-SSCP analysis for the (A)_9 track of the hMSH3 gene in these cases. Shifted bands are detected in tumor DNAs. (D) One base deletion in the (A)_8 track of the hMSH3 gene confirmed by DNA sequencing. A representative result of one base deletion in tumor DNA is shown (case 22).

Table II. Incidence of RER in sporadic and familial gastric cancers

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>RER</th>
<th>Incidence</th>
<th>No. of loci examined</th>
<th>Reference</th>
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<tr>
<td>Sporadic</td>
<td>≥ 1 locus</td>
<td>22/57 (39)*</td>
<td>2</td>
<td>(23)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16/52 (31)</td>
<td>5</td>
<td>(24)</td>
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<td></td>
<td>5/22 (23)</td>
<td>10</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20/76 (26)</td>
<td>2</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11/46 (24)</td>
<td>8</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11/34 (32)</td>
<td>12</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10/42 (24)</td>
<td>4</td>
<td>(29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/18 (18)</td>
<td>7</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6/40 (15)</td>
<td>3</td>
<td>(31)</td>
</tr>
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<td></td>
<td></td>
<td>4/26 (15)</td>
<td>6</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>≥ 2 loci</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>0/5 (0)</td>
<td>22</td>
<td>(7)</td>
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<td></td>
<td>4/6 (67)</td>
<td>8</td>
<td>(6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14/31 (45)</td>
<td>7</td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7/28 (25)</td>
<td>6</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2/4 (50)</td>
<td>7</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9/31 (29)</td>
<td>7</td>
<td>(18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5/28 (18)</td>
<td>6</td>
<td>this study</td>
</tr>
<tr>
<td>Familial</td>
<td>≥ 1 locus</td>
<td>0/3 (0)</td>
<td>22</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/6 (67)</td>
<td>8</td>
<td>(6)</td>
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<td></td>
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<td>9/31 (29)</td>
<td>7</td>
<td>(18)</td>
</tr>
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<td></td>
<td></td>
<td>5/28 (18)</td>
<td>6</td>
<td>this study</td>
</tr>
</tbody>
</table>

*aNumbers in parentheses, percentages.
the inactivation of this receptor may be an important step in the tumorigenesis (36). In gastric cancer with RER phenotype, TGF-β RII mutations have been reported in one of two (50%) familial cases (6), five of seven (71%) sporadic cases (10) and 10 of 11 (91%) widespread RER cases (34). In this study, we detected three of four (75%) cases with more than three RER. Recently, Konishi et al. (37) have shown high mutation rates of this repetitive sequence in the TGF-β RII gene in HNPCC with severe RER, moderate rates in non-HNPCC with severe RER, and no mutations in non-HNPCC with mild RER, suggesting that inactivation of the TGF-β RII gene is one of the important genetic events associated with the severe RER phenotype. The present result of no TGF-β RII mutation in cases with less than two RER, supports the hypothesis that the TGF-β RII gene is also a target gene for severe RER phenotype in FGC.

A mutation of the (GT)3 repeat in the TGF-β RII gene has been reported in a colon cancer cell line (9,11). Our PCR primers were designed to amplify the region of two (GT)3 repeats and a part of the Ser/Thr kinase domain. No mutation was detected, suggesting that the alteration in the small region containing the (GT)3 repeats in the TGF-β RII gene is not associated with the development of FGC.

Recently a model of DNA mismatch repair system with MSH2/MSH3/MSH6 was proposed (38–40). There were two alternative pathways of the MSH2 dependent mismatch repair system: MSH2 and MSH6 for single base mismatch, and MSH2 and MSH3 or MSH2 and MSH6 for insertion/deletion mutations. In human, frameshift mutations of the (A)n or (C)n sequences in the hMSH3 and hMSH6 genes, respectively, occur in various cancers and frequently in colon cancers with RER phenotype (12). This study also showed the frequent hMSH3 frameshift mutation in gastric cancers with RER at more than three loci. However, it is not clear whether frameshift mutations in these genes are generated by the defect of other mismatch repair genes, such as hMSH2 and hMLH1, and is a consequence of mutagenic actions in the tumor with RER phenotype. Although some hMSH3/hMSH6 double mutants were shown previously (41), no frameshift mutations were detected in the (C)n sequence of the hMSH6 gene in our cases, indicating that hMSH3-rat RER is rare in gastric cancer and hMSH3/hMSH6 double mutation is not common in FGC.

Four severe RER cases with frameshift mutation in the TGF-β RII and/or hMSH3 gene had no colorectal cancer cases in their families, indicating that these were not typical cases of HNPCC.

In conclusion, molecular analysis of a large number of FGCs revealed that the RER phenotype itself does not seem to be specifically characteristic of the familial aggregation of gastric cancer in general, indicating that familial clustering of gastric cancer is etiologically distinct from HNPCC.

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