Analyses of mutation and loss of heterozygosity of coding sequences of the entire transforming growth factor beta type II receptor gene in sporadic human gastric cancer

Rong-Jun Guo1,2, Ying Wang1, Eizo Kaneko2, Dong-Yu Wang1, Hajime Arai3, Hiroyuki Hanai2, Seiichi Takenoshiba1,4, Koichi Hagijwara4, Curtis C.Harris4 and Haruhiko Sugimura1,5

1First Department of Pathology and 2First Department of Internal Medicine, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu, Shizuoka 431-31, 3First Department of Surgery, Gunma University School of Medicine, Maebashi, Gunma 371, Japan and 4Laboratory of Human Carcinogenesis, NCI, NIH, Bethesda, MD 20892, USA

Mutations in the transforming growth factor beta type II receptor (TGFβRII) gene have been detected in several human cancer types exhibiting microsatellite instability. Using intron primers previously reported for examination of the entire coding region of the TGFβRII gene, 29 sporadic gastric cancers were screened with non-radioactive single strand conformation polymorphism and subsequent DNA sequencing analysis. Mutations of the TGFβRII gene were detected in three out of 29 tumors (10%). Two cases showed deletions in a polyadenine tract in both alleles and was positively associated with replication error. One case had an insertion of GA dinucleotide sequence in one allele. Mutations of the TGFβRII gene were restricted to exon 3 and other coding regions were not affected. Loss of heterozygosity was detected by analyzing a polymorphic site in intron 2. Three out of nine (33%) informative cases, which were all of intestinal type and advanced cases, showed loss of heterozygosity but neither TGFβRII mutation nor replication error was found in these cases. Immunoreactivity of TGFβRII in tumor tissues was reduced to a different extent in the gastric cancer with genetically abnormal transforming growth factor. Although the numbers studied are small, homozygous (A)30 deletion or loss of heterozygosity of TGFβRII is involved in tumorigenesis and progression of at least some part of sporadic gastric cancer.

Introduction

Mutations in the transforming growth factor beta type II receptor (TGFβRII) gene have been found in various human cancers, including colon (1–4), stomach (5), and head and neck (6). These and other observations are consistent with the hypothesis that TGFβRII is a tumor suppressor gene whose mutational inactivation is important in human carcinogenesis (7,8). Mutations in the TGFβRII gene found to date cluster in domains IX and XI (codons 505–544) of the kinase region (3,6,9) and in two microsatellites at codons 125–128 (A)10 and 532–534 (GT)2 in colon, gastric and endometrial cancers with microsatellite instability (3,5). Most studies have analyzed either cancer cell lines or frozen tumor series that can generate cDNA for polymerase chain reaction (PCR)-based analysis. Mutation analysis of genomic level in primary tumors is indispensable to fully understand the biological significance of TGFβRII in carcinogenesis, and intron-based primers for mutation analysis of genomic DNA have become available recently (10,11). We used these reagents to screen genomic DNA samples from 30 sporadic colorectal cancers for TGFβRII mutations. We found a case with a deletion in a polyadenine tract in exon 3 and a point mutation in the kinase domain located in exon 7; both cases displayed a replication error (RER)-positive phenotype (4). By analyzing genomic DNA, ‘two-hit’ inactivation of the TGFβRII gene, deletion or insertion in (A)10 tract and another mutation in a different allele have also been reported in colon tumors from patients with hereditary non-polyposis colorectal cancer (HNPCC) (12). Accumulating evidence indicates that different kinds of cancers have characteristic patterns of mutations in the TGFβRII gene. Its mutation has been mostly associated with and is present in >90% of cases of a subgroup of colon cancers with RER (1,3,13–15). RER-positive colon cancers characterizedly inactivate TGFβRII by acquiring insertions or deletions of one or two adenine base pairs in this repeat tract of adenes (1). It is also reported (5) that these same TGFβRII frameshift mutations were common in RER positive gastric cancers (five of seven cases) but were less common in RER-positive endometrial cancers (four of 24 cases); Ouyang et al. (16) also reported that although the incidence of RER was high in pancreatic cancer, no mutations in the TGFβRII were found, thereby suggesting that tissue-specific factors play a role in determining the generation or selection for these mutations (5). Moreover, inactivation of TGFβRII has been reported in non-RER gastric cancer cell lines via gene deletion (17).

Because previous investigations on the mutation of TGFβRII in gastric cancer have been based on analyses of cDNA level or genomic level, but have only focused on some particular regions, we thought it worthy to analyze the entire coding region at the genomic level to fully understand the manifestations and mechanisms of TGFβRII in gastric carcinogenesis. In this study, 29 sporadic gastric cancers were analyzed by PCR–single strand conformation polymorphism (SSCP) and DNA sequencing for mutations in the TGFβRII gene in genomic DNA samples, and we investigated the relationships with RER, loss of heterozygosity (LOH) and immunohistochemical findings.

Materials and methods

Tissue samples

Primary gastric cancers and corresponding normal tissues were obtained from 29 patients resected at the affiliated hospitals of Hamamatsu University School of Medicine, Shizuoka, Japan. The histological types include 10 well differentiated, seven moderately differentiated and eight poorly differentiated.
adenocarcinomas, three signet ring cell adenocarcinomas and one mucinous adenocarcinoma, which were diagnosed by WHO criteria (18). Twenty-one tumors were of advanced stage with regional lymph nodes or distant metastases. Genomic DNA samples were isolated by proteinase K treatment and phenol chloroform extraction using standard protocols (19).

PCR–SSCP analysis

All samples were examined for mutations in exons 1–7 of the TGFβRII gene by PCR–SSCP analysis. Using the intron primers designed by Takenoshita et al. (4), PCR was accomplished with 50 ng of genomic DNA suspended in a total volume of 25 μl PCR reaction buffer containing 1× Ex buffer, 0.1 mM deoxynucleotide triphosphate, 0.1 mM Mg(OAC)₂, 0.1 mM primer and 0.5 U Takara Ex Taq DNA Polymerase (Takara, Osaka, Japan). The PCR conditions were as follows; 40 s at 94°C, 30 s at 55°C or 60°C, and 90 s at 68°C for 35 cycles, followed by 8 min at 68°C. Aliquots of 5–10 μl of each PCR product were then denatured, cooled on ice, loaded on neutral 8–10% polyacrylamide gels with and without 5% (vol/vol) glycerol, electrophoresed at room temperature, stained with 0.1% amido/7.6 M gels as described previously (20).

Sequence analysis

DNA fragments with mobility shifts or extra bands were sequenced. Amplified DNA fragments were first purified by phenol–chloroform extraction and ethanol precipitation, loaded on 2% agarose gel electrophoresed at room temperature and further purified using a Quagel II Gel Extraction Kit (Quagen, Chatsworth, CA) and then cloned into the pGEM-T Vector using the TA Cloning System (Promega, Madison, WI) according to the manufacturer’s protocol. Sequencing reactions were performed using the T7 Sequenase DNA polymerase and 7-deaza-dGTP (Amersham Life Science, Arlington Heights, IL). The sequence reaction samples were loaded on 6% denaturing polyacrylamide gels, electrophoresed, dried, and exposed to Kodak XAR film for 20–24 h at room temperature.

To circumvent a ‘false’ identification of mutations caused by misincorporation by polymerase, we duplicated PCR and SSCP in all cases. More than four clones were sequenced. When we could have two or more consistent mutated clones confirmed by sequencing bidirectionally, we defined this case had a mutation.

Analysis of microsatellite instability (MI)

MI was determined using five or more of the microsatellite markers (at D1S116, D2S136, D5S82, D6S87, D17S261 and TP53). In each case, one of the primers was 32P end-labeled, and paired DNAs of normal and cancerous tissues were amplified by PCR followed by electrophoresis in 5% polyacrylamide/7.6 M gels as described previously (21). Nucleotide sequences of the primers and detailed conditions for PCR amplification of microsatellites have been reported elsewhere (4,21). Tumors with new bands at two or more of the microsatellite loci were defined as RER positive.

Loss of heterozygosity (LOH) analysis

LOH was evaluated by analyzing a frequent polymorphic site within intron 2 (12). The exon 2 PCR product revealed an identifiable polymorphism by SSCP (Figure 2). The sequencing analyses of case A (lane 3), case B (lanes 1 and 2) and wild-type (lane 4) in exon 3. Lane N represents (A)10 wild-type. (C) Sequence analysis of case C (right), which shows G:A insertion just upstream of (A)10 compared with the wild-type sequence (left).

Results

Mutation analysis

We examined all TGFβRII exons in 29 gastric cancers. Three cases gave a band shift in SSCP. Two had a deletion in the (A)10 repeat: case A was a deletion of a single A and case B was two deletions of two and three adenines (Figure 1). Both cases had microsatellite instability. Both cases had mutations of the TGFβRII gene in both alleles: in case A, the intensity of a band corresponding to the wild-type allele was very faint in PCR–SSCP, which was presumably contributed by contaminating normal cells. In this case, the extra band was clearly visible. In case B, two extra bands were clearly visible while the band corresponding to the wild-type sequence was also visible. In case C, an extra band was visible (8% polyacrylamide with 5% glycerol at room temperature). (B) Sequencing analyses of case A (lane 3), case B (lanes 1 and 2) and wild-type (lane 4) in exon 3. Lane N represents (A)10 wild-type. (C) Sequence analysis of case C (right), which shows G:A insertion just upstream of (A)10 compared with the wild-type sequence (left).

Immunohistochemistry

Immunohistochemical identification of TGFβRII and cyclin D1 in cancer and non-cancerous portions of resected specimens were performed. Polyclonal antibodies L21 and H567, which recognize amino acid 246–266 and 1–567 of TGFβRII respectively (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal antibody cyclin D1 (clone DCS-6) (Novocasta, Newcastle, UK) were applied to microwaved dewaxed tissues. Specific immunoreactivity was detected with a streptavidin–biotin complex peroxidase system using diaminobenzidine as substrate as described elsewhere (22). The sections were then counterstained with hematoxylin. Cytoplasmic staining of L21 for TGFβRII was considered positive as reported (23) and nuclear staining of cyclin D1 as seen in follicular lymphoma (positive control) was defined as positive. Antibody H567 was immunoreactive in cytoplasmic and nuclear regions of the epithelium.

Polyorphism analysis

Three patterns of bands were noticed in exon 2 SSCP (Figure 2). Sequencing showed that an a/g polymorphism at the seventh
Recent studies have provided evidence that is consistent with the hypothesis that the TGFβRII gene is a tumor suppressor in several types of human cancers (1–3,8), and it is generally believed that its inactivation is tightly associated with RER.

In this report, we examined the genomic status of TGFβRII in primary gastric carcinoma. One of our aims was to see whether there is any mutation that affects TGFβRII function outside the (A) repeat. We also wanted to see whether the so-called two-hit mechanism outside the (A) repeat was involved in inactivation of TGFβRII in gastric cancers, as base of intron 2 was responsible for the polymorphism and the three types found in SSCP were a/a, a/g and g/g as observed by Lu et al. (12). Among the 29 cases of gastric cancers, six were a/a types (21%), nine a/g types (31%) and 14 g/g types (48%) respectively. Three of the nine informative cases (a/g type) showed LOH of the TGFβRII (50%). These results were consistent with the results for gastric cancer reported previously. One case (case C), which showed only one MI, had a G:A dinucleotide insertion just upstream of the polyadenine tract. In our data set, all the cancers with MI and/or associated (A) deletion [four out of four cases with MI positive and two out of two cases with (A) deletion] were located in the lower part of the stomach (Table I). But the case with G:A deletion, which showed only one MI, was located in the proximal part of the stomach, and this may indicate a different mechanism of carcinogenesis.

Table II. Relationship between mutation of TGFβRII and immunohistochemistry of TGFβRII and cyclin D1

<table>
<thead>
<tr>
<th>Genetic alteration of TGFβRII</th>
<th>Cyclin D1+</th>
<th>L21</th>
<th>H567</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case A Homozygous mutation</td>
<td>−/+ b</td>
<td>−/+ 70% c</td>
<td>−/+ 70% c</td>
</tr>
<tr>
<td>Case B Homozygous mutation</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>Case C Heterozygous mutation</td>
<td>−/+</td>
<td>−/+ 10% d</td>
<td>±10% d</td>
</tr>
<tr>
<td>(GA insertion)</td>
<td>+ 70%</td>
<td>+ 90%</td>
<td></td>
</tr>
<tr>
<td>Case D LOH</td>
<td>−/+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Case E LOH</td>
<td>−/+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Case F LOH</td>
<td>−/+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

aCyclin D1, L21 and H567 represent antibodies used in this study.
bImmunoreactivity is graded as −/+ (negative or faint), + (moderately positive), ++ (equal to positive control), +++ (equal to positive control) or +++++ (overexpression).
c|d|e|f|g|h

Microsatellite instability
Of the 29 cases, four were RER positive (14%) at two or more loci. Among them, two cases showed deletions in the polyadenine (A) repeat of the TGFβRII (50%). These results were consistent with the results for gastric cancer reported previously. One case (case C), which showed only one MI, had a G:A dinucleotide insertion just upstream of the polyadenine tract. In our data set, all the cancers with MI and/or associated (A) deletion [four out of four cases with MI positive and two out of two cases with (A) deletion] were located in the lower part of the stomach (Table I). But the case with G:A deletion, which showed only one MI, was located in the proximal part of the stomach, and this may indicate a different mechanism of carcinogenesis.

Immunohistochemistry of TGFβRII
The fundic glands of the stomach were always immunoreactive and were consequently used as a positive control. Nontumorous gastric foveolar epithelium was weakly positive for L21 and H567. We compared these mutations found in our series with immunologically identifiable inactivation or down-regulation of TGFβRII expression in gastric cancer cells. In cases with TGFβRII mutations, the inactivation to TGFβRII recognized by L-21 and H567 was reduced to a different extent (Table II). In cases with (A) deletion in the proximal part of the stomach (Table I). But the case with G:A deletion, which showed only one MI, was located in the proximal part of the stomach, and this may indicate a different mechanism of carcinogenesis. 

Discussion
Recent studies have provided evidence that is consistent with the hypothesis that the TGFβRII gene is a tumor suppressor in several types of human cancers (1–3,8), and it is generally believed that its inactivation is tightly associated with RER.

In this report, we examined the genomic status of TGFβRII in primary gastric carcinoma. One of our aims was to see whether there is any mutation that affects TGFβRII function outside the (A) repeat. We also wanted to see whether the so-called two-hit mechanism outside the (A) repeat was involved in inactivation of TGFβRII in gastric cancers, as shown in Table I. The three types found in SSCP were a/a, a/g and g/g as observed by Lu et al. (12). Among the 29 cases of gastric cancers, six were a/a types (21%), nine a/g types (31%) and 14 g/g types (48%) respectively. Three of the nine informative cases (a/g type) showed LOH of the TGFβRII (50%). These results were consistent with the results for gastric cancer reported previously. One case (case C), which showed only one MI, had a G:A dinucleotide insertion just upstream of the polyadenine tract. In our data set, all the cancers with MI and/or associated (A) deletion [four out of four cases with MI positive and two out of two cases with (A) deletion] were located in the lower part of the stomach (Table I). But the case with G:A deletion, which showed only one MI, was located in the proximal part of the stomach, and this may indicate a different mechanism of carcinogenesis.

Table I. Comparison of RER and the mutations and LOH of TGFβRII with clinical and histological characteristics

<table>
<thead>
<tr>
<th>Clinical and histological classification</th>
<th>RER+</th>
<th>RER−</th>
<th>Mutations of TGFβRII</th>
<th>LOH of TGFβRII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology: well-differentiated</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>moderately differentiated</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>poorly differentiated</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Stage: early</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>advanced</td>
<td>2</td>
<td>19</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Location: upper</td>
<td>0</td>
<td>13</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>middle</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>lower</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Lauren’s classification: intestinal type</td>
<td>3</td>
<td>16</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>diffuse type</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

aNo overlapping between mutations and LOH of TGFβRII gene.
reported in HNPCC (12). Previous studies on the TGFβRII mutation in gastric cancer have been based on the analysis of cDNA products or genomic DNA, and have only focused on some particular regions. To further characterize the role of the TGFβRII gene in carcinogenesis of sporadic gastric cancer, we analyzed the entire coding sequence of the TGFβRII gene in 29 cases of sporadic Japanese primary gastric cancer by using previously designed intron-based primers (4).

At first, as with most investigators, we attempted to use DNA samples extracted from paraffin-embedded tissues because of the rich available resources, but this kind of DNA sample was often unsuccessful for amplification by most of our designed intron primers. Therefore, we had to use the limited DNA samples extracted from frozen tissues.

In this study, three cases were found to have a frameshift mutation in the \((A)_{10}\) repeat tract of TGFβRII (10%), but no mutation was found outside this region, which indicates that the \((A)_{10}\) repeat tract is the main target of TGFβRII mutation in sporadic gastric cancer: other coding sequences are rarely affected. Moreover, two of three cases had mutations in the \((A)_{10}\) repeat tract of both alleles, which suggests that the homozygous mutations (mutations in both alleles) of TGFβRII are common in the carcinogenesis of sporadic gastric cancer. Lu et al. reported frequent genetic changes in both alleles in HNPPC (12) [heterozygous deletions in the \((A)_{10}\) repeat of the TGFβRII gene and an additional missense mutation]. They described this heterozygous deletion in the \((A)_{10}\) repeat plus somatic mutation as two-hit inactivation. In our series, homozygous deletion in the \((A)_{10}\) repeat tract was frequently found and may reflect another two-step inactivation. In case B, sequencing showed that one allele had 3-A deletions in the \((A)_{10}\) repeat tract, which has not been reported previously.

For the relationship between mutation of TGFβRII and RER, we found four of 29 cases with RER+ (14%) based on our criteria, and among them two cases had frameshift mutations in the \((A)_{10}\) repeat tract (50%). Seemingly, incidence of RER+ and its related TGFβRII mutation was lower than that reported by others (15–38%) (5,24–27). We presume these discrepancies might result from different RER criteria, and selection of number and types of microsatellite markers, patient and cancer types, and DNA origin (from paraffin or frozen tissue), etc. For example, as far as RER+ criteria are concerned, some researchers have chosen \(\geq 1\) loci microsatellite marker that showed MI as RER+ (24,25), but recent study has indicated only one locus or even two loci, which shows that MI is not an appropriate indicator of mutator phenotype criteria (27). Therefore, some investigators chose eight of 11 loci that showed MI as a phenotype mutator criterion (27). We adopted \(\geq 2\) loci (out of four loci) that showed MI as a RER+ criterion. We think our criteria, DNA origin and case selection, are close to that of Nakashima et al.’s report (26). Our RER+ incidence was similar to theirs (15%). However, these criteria might risk missing some cases that have a real mutator phenotype. In our case C, which showed a GA dinucleotide addition just upstream of \((A)_{10}\), only one locus had MI, and this might be considered a mutator phenotype that escaped our criteria. If this case is included as an RER+ category, the RER+ incidence should be 17%, and three of five cases with RER+ showed frameshift mutations in the \((A)_{10}\) repeat tract of TGFβRII (60%). On the other hand, in our RER+ without mutation of TGFβRII, there is a possibility that some of them might not be genuine mutator phenotypes as indicated in Chung’s report (27). Alternately, we cannot exclude the possibility that there may be another mutational mechanism for the genetic change in addition to deficient mismatch repair (MMR) in case C.

The prevalence of the a/g polymorphism in intron 2 of the TGFβ II receptor has not been previously described. Although the biological significance of this polymorphism is unclear, the distribution in gastric cancer in our series was different from that in healthy controls (data not shown), although rigorous statistical analysis will require more cases.

The TGFβRII gene has been mapped to chromosome 3p22 (28), where deletions commonly occur in several tumors
polymorphism inside the TGF-βIIR gene, we found LOH in three of nine informative cases, all of which were at an advanced stage and histologically classified as intestinal type, which indicates that LOH in the TGF-βIIR might be associated with progression of gastric cancer rather than an early event in gastric carcinogenesis. In many tumor suppressor genes, one allele is inactivated by deletion and the other allele is inactivated by point mutation. However, we could not observe this mode of inactivation in TGF-βIIR because mutation was not detected in the remaining allele in the cases with LOH.

We compared these mutations and LOH of TGF-βIIR found in our series with immunologically identifiable inactivation or downregulation of TGF-βIIR expression in gastric cancer cells. In cases with TGF-βIIR mutations, the immunoreactivity to TGF-βIIR recognized by L-21 and HS67 was reduced to a different extent (Table II) but did not completely disappear. In case C, GA insertion had been also expected to generate a truncated protein, but the reduction in immunoreactivity was not remarkable. In this case, the remaining wild-type allele was present and this may explain why the reduction of immunoreactivity was not dramatically detected. Another explanation could be the possible heterogeneity of TGF-βIIR mutation in the tumor tissue. The heterogeneity of immunoreactivity in the tumor is also consistent with this idea. Alternatively there might also be a cross-reactivity to the other cellular proteins. The lists of proteins that have homology with the immunogenes used (data not shown) include other kinds of receptor protein kinase that are known to be overexpressed in gastric cancer, although they did not investigate the entire coding sequence, show one of the characteristics of human gastric cancer.

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