Mutation and Expression of DNA2 Gene in Gastric and Colorectal Carcinomas

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Background : Deregulation of DNA repair and replication are involved in cancer development. DNA2 is a nuclease/helicase that plays roles in DNA repair and replication. The aim of this study was to explore DNA2 mutation and DNA2 protein expression in gastric cancers (GCs) and colorectal cancers (CRCs). Methods : We analyzed two mononucleotide repeats in DNA2 in 27 GCs with high microsatellite instability (MSI-H), 34 GCs with stable MSI (MSS), 29 CRCs with MSI-H and 35 CRCs with MSS by single-strand conformation polymorphism. We also analyzed DNA2 expression in GCs and CRCs either with MSI-H or MSS. Results : We found DNA2 mutations in two GCs (7.1%) and two CRCs with MSI-H (6.9%), but not in cancers with MSS. The mutations consisted of three cases of a c.2593delT and one of a c.2592_2593delTT, which would result in premature stopping of amino acid synthesis (p.Ser865Hisfsx6 and p.Ser865Thrfsx20, respectively). DNA2 expression was observed in 16 (80%) of the GCs and 15 (75%) of the CRCs with MSI-H, but all of the cancers with DNA2 frameshift mutations were weak or negative for DNA2. Conclusions : Our data indicate that DNA2 mutation and loss of DNA2 expression occur in GCs and CRCs, and suggest that these alterations may contribute to cancer pathogenesis by deregulating DNA repair and replication.

Key Words : Stomach neoplasms; Colonic neoplasms; DNA2; Mutation; Immunohistochemistry
vate the function of DNA2 in the cancer cells. In the present study, we analyzed T9 and A7 within DNA2 in GCs and CRCs with MSI. We also analyzed expression of DNA2 protein in GCs and CRCs.

MATERIALS AND METHODS

Mutational analysis

Methacarn-fixed tissues of 61 GCs and 64 CRCs were used for this study. All of the patients with cancers were Koreans. The cancers consisted of 27 GCs with high MSI (MSI-H), 34 GCs with stable MSI (MSS), 29 CRCs with MSI-H, and 35 CRCs with MSS (according to the National Cancer Institute criteria).23 The GCs with MSI-H consisted of 15 diffuse-type and 12 intestinal-type carcinomas (by Lauren’s classification), while the GCs with MSS consisted of 20 diffuse-type and 14 intestinal-type carcinomas. The GCs with MSI-H consisted of two early GCs (EGCs) and 25 advanced GCs (AGCs), while the GCs with MSS consisted of two EGCs and 32 AGCs. The tumor, node and metastasis (TNM) stages of the GCs with MSI-H were twelve stage I, nine stage II, five stage III and one stage IV, while those of the GCs with MSS were nine stage I, fifteen stage II, seven stage III and three stage IV. The CRCs originated from cecum (MSI-H \(n = 5\), MSS \(n = 2\)), ascending colon (MSI-H \(n = 17\), MSS \(n = 2\)), transverse colon (MSI-H \(n = 7\), MSS \(n = 0\)), sigmoid colon (MSI-H \(n = 0\), MSS \(n = 12\)) and rectum (MSI-H \(n = 0\), MSS \(n = 19\)). The TNM stages of the CRCs with MSI-H were five stage I, ten stage II, twelve stage III and two stage IV, while those of the CRCs with MSS were five stage I, thirteen stage II, fifteen stage III and two stage IV. Malignant cells and normal cells were selectively procured from hematoxylin and eosin-stained slides using a 30 G1/2 hypodermic needle by microdissection as described previously.34,25

Exon 15 (T9) and exon 11 (A7) of DNA2 have mononucleotide repeats. Genomic DNA from microdissected cells was isolated, and amplified by polymerase chain reaction (PCR) with specific primer pairs. Forward and reverse that could amplify exon 15, were, respectively: \(5\prime-\text{CATCCAAATATTTTCCCG-TAA}^{-3}\prime\) and \(5\prime-\text{AAGTGCTCTGCSCTACCCGCS-GS}^{-3}\prime\). Pairs that amplify exon 11 were: \(5\prime-\text{GAACTTGTCCGGTCTCGTCC-3}\prime\) and \(5\prime-\text{AATTCAAAATTTGGCTCTATTGT}^{-3}\prime\). Each PCR reaction was done under standard conditions in an 8 µL reaction mixture. Radioisotope \((^{32}\text{P})\text{dCTP}\) was incorporated into PCR products for detection by single-strand conformation polymorphism (SSCP) autoradiograms. The reaction mixture was denatured for 1 minute at 94°C and incubated for 30 cycles (denaturing for 40 seconds at 94°C, annealing for 40 seconds at 50-60°C, and extending for 40 seconds at 72°C). Final extension was continued for 5 minutes at 72°C. After amplification, PCR products were denatured 5 minutes at 95°C at a 1 : 1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH and were loaded onto an MDE gel (Cambrex Bio Science Rockland, Rockland, ME, USA) with 10% glycerol. Then, PCR products were electrophoresed at 8 W at room temperature overnight. After electrophoresis, the gels were transferred to 3-mm Whatman paper and dried, and autoradiography was done. Mobility shifts on the SSCP were determined by visual inspection. Direct DNA sequencing reactions were done in the cancers with the mobility shifts in the SSCP. Sequencing of the PCR products was carried out using a capillary automatic sequencer (3730 DNA Analyzer, Applied Biosystem, Carlsbad, CA, USA) according to the manufacturer’s recommendation.

Immunohistochemistry

Using the sections from GCs and CRCs tissues, immunohistochemistry for DNA2 was done. The tissues consisted of 20 GCs and 20 CRCs with MSI-H, and 20 GCs and 20 CRCs with MSS. For immunohistochemistry, we used DAKO REAL Envision System (DAKO, Glostrup, Denmark) with a rabbit polyclonal antibody against human DNA2 (Abcam, Cambridge, UK). This antibody was raised by a peptide immunogen within the amino acids 960-1,060. After deparaffinization, heat-induced epitope retrieval was done by immersing slides in Coplin jars filled with 10 mmol/L citrate buffer (pH 6.0). After epitope retrieval, slides were treated with 1% H2O2 in phosphate buffered saline for 15 minutes at room temperature to abolish endogenous peroxidase activity. After washing with TNT buffer (0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl and 0.05% Tween 20) for 20 minutes, slides were treated with TNB buffer (0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, and 0.5% blocking reagent). Sections were incubated overnight at 4°C with DNA2 antibody (1 : 200). They were then incubated with peroxidase-labeled polymer that was conjugated with secondary antibody for 30 minutes. Each incubation step was followed by three washes for 5 minutes in TNT buffer. The reaction products were developed with diaminobenzidine (Sigma, St. Louis, MO, USA) and counterstained with hematoxylin. Tumors were interpreted as positive by immunohistochemistry when the cancer cells showed an intense immunostaining. The results were
reviewed independently by two pathologists. As negative controls, slides were treated by replacement of the primary antibody with the blocking reagent.

RESULTS

Mutations of DNA2

Genomic DNAs isolated from normal and tumor tissues of the 61 GCs and 64 CRCs through microdissection were analyzed for detection of mutations in the DNA2 gene (exon 11 and 15) by PCR-SSCP assay. Overall, PCR-SSCP analysis identified aberrant bands in 4 (3.2%) of the 125 cancers analyzed. None of the corresponding normal samples showed evidence of mutations by SSCP, indicating that the aberrant bands had risen somatically (Fig. 1A). Direct DNA sequencing analysis of the cancers with the aberrant bands in the SSCP led to identification of four DNA2 frameshift mutations in exon 15, but none in exon 11 (Table 1, Fig. 1B). The mutations consisted of three c.2593delT and one c.2592_2593delTT, which would result in premature stops of amino acid synthesis (p.Ser865Hisfsx6 and p.Ser865Thrfsx20, respectively). All of the mutations were detected in cancers with MSI-H (7.1%, 4/56) (Table 1), and not in those with MSS (0%, 0/69). Mutations were found in 2 of 27 GCs with MSI-H (7.4%) and 2 of 29 CRCs with MSI-H (6.9%).

We carefully reviewed the clinicopathologic data (age, sex, histologic grade, stage, and metastasis), but there was no significant association of DNA2 mutations with any of these parameters. There was also no correlation between histological features of the tumors and the presence of DNA2 mutations. To

<table>
<thead>
<tr>
<th>Location</th>
<th>Repeats (wild type)</th>
<th>Repeats (mutation)</th>
<th>Incidence in MSI-H cancers (%)</th>
<th>Nucleotide change (predicted amino acid change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 15</td>
<td>T9</td>
<td>T8</td>
<td>Gastric: 1/27 (3.7)</td>
<td>c.2593delT (p.Ser865Hisfsx6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colorectal: 2/29 (6.9)</td>
<td></td>
</tr>
<tr>
<td>Exon 15</td>
<td>T9</td>
<td>T7</td>
<td>Gastric: 1/27 (3.7)</td>
<td>c.2592_2593delTT (p.Ser865Thrfsx20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colorectal: 0/29 (0)</td>
<td></td>
</tr>
<tr>
<td>Exon 11</td>
<td>A7</td>
<td>No mutation</td>
<td>Gastric: 0/27 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colorectal: 0/29 (0)</td>
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MSI-H, high microsatellite instability.
confirm the mutation data, we repeated the PCR-SSCP twice. In the second round of the SSCP, we included a positive control that had been detected in the first round of SSCP, and found that the data were consistent (data not shown). There was a significant difference in the DNA2 mutation frequency between the MSI-H (4/56) and non-MSS (0/69) cancers (Fisher’s exact test, p = 0.038).

**Expression of DNA2**

We analyzed, by immunohistochemistry, DNA2 protein expression in 20 GCs and 20 CRCs with MSI-H, and 20 GCs and 20 CRCs with MSS. The 40 cancers with MSI-H included four cancers with DNA2 mutations and 36 cancers without such mutations. In the cancers with MSI-H, immunopositivity for DNA2 was observed in 16 (80%) of the GCs and 15 (75%) of the CRCs (Table 2). Of the four cancers with DNA2 frameshift mutations (two GCs and two CRCs), all of them showed very weak or negative DNA2 immunostaining (Fig. 2). The remain-

<table>
<thead>
<tr>
<th>No. of cancers with DNA2 expression (%)</th>
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<tbody>
<tr>
<td>GCs with MSI-H (n = 20)</td>
</tr>
<tr>
<td>CRCs with MSI-H (n = 20)</td>
</tr>
<tr>
<td>GCs with MSS (n = 20)</td>
</tr>
<tr>
<td>CRCs with MSS (n = 20)</td>
</tr>
<tr>
<td>MSI-H GCs and CRCs with DNA2 mutation (n = 4)</td>
</tr>
<tr>
<td>MSI-H GCs and CRCs without DNA2 mutation (n = 36)</td>
</tr>
</tbody>
</table>

GCs, gastric cancers; MSI-H, high microsatellite instability; CRCs, colorectal cancers; MSS, stable MSI.

**Table 2. Summary of DNA2 expression in gastric and colorectal cancers**

![Fig. 2. Visualization of DNA2 expression in gastric and colorectal cancer tissues by immunohistochemistry.](image-url)
ing 36 cancers with MSI-H and without DNA2 mutations showed DNA2 expression in 31 cancers (86%). There was a significant difference in DNA2 immunostaining between MSI-H cancers with DNA2 frameshift mutations and those without the mutations (Fisher’s exact test, p = 0.001). In the cancers with MSS, immunopositivity for DNA2 was observed in 17 (85%) of the GCs and 18 (90%) of the CRCs (Table 2). There was no significant difference in DNA2 immunopositivity between the cancers with MSI-H and MSS (Fisher’s exact test, p > 0.05). The immunostaining of DNA2, when present, was observed mainly in nuclei, and only weakly in the cytoplasm (Fig. 2). Negative controls using the blocking solution instead of the primary antibody showed no signal.

**DISCUSSION**

Despite earlier intensive work that discovered many frameshift mutations in cancers with MSI,\(^{11-23}\) it appears that many frameshift mutations in cancers still remain to be discovered. Frequent alterations in cell cycle and DNA damage signaling/repair-related genes and their products in cancers\(^ {17-20}\) led us to analyze the DNA2 gene for detection of somatic mutations in GCs and CRCs. Because mononucleotide repeats are frequent targets for somatic mutations in GCs and CRCs with MSI,\(^ {11-23}\) we focused the analysis within the mononucleotide repeats of DNA2. We found that the DNA2 gene harbored four somatic frameshift mutations within the mononucleotide repeats in the coding sequences. These mutations were found in the cancers with MSI-H, but not in those with MSS, indicating that association of the mutations with MSI-H is specific. Also, we analyzed tissue expression of DNA2 protein in cancers with MSI-H and MSS by immunohistochemistry. We found that DNA2 was not expressed in 20-25% of the cancers with MSI-H, and 10-15% of those with MSS. Together, these data indicate that the DNA2 gene is altered in some GCs and CRCs by somatic mutation and/or loss of expression.

DNA2 immunostaining was not detected or only weakly detected in the four cancers with DNA2 frameshift mutations. Because the anti-DNA2 antibody was raised by a peptide immunogen within the amino acid sequence (960-1,060) that would be removed by a frameshift mutation (p.Ser865Hisfsx6 and p.Ser865Thrfsx20), the mutated DNA2 proteins could not be detected by the antibody. Loss of DNA2 immunostaining in the cancers with the DNA2 mutations might be caused by a frameshift mutation in one allele and by other gene silencing mechanisms in the second allele. Another possibility for absence of immunoreactivity is that the quantity of DNA2 expression from one allele might not be enough to be detected by the antibody in the immunohistochemistry assay. We also found that DNA2 expression was down-regulated not only in the cancers with mutations, but also in those without the mutations, suggesting the possibility that loss of expression may be regulated by other mechanisms besides frameshift mutations.

There have been debates on the intracellular location of the DNA2 protein. An earlier study reported that DNA2 was localized to mitochondria,\(^ 6\) whereas another study showed that it was localized to both nuclei and mitochondria.\(^ 5\) We observed that DNA2 expression was evident in both cytoplasm and nuclei, although it was weak in cytoplasm and much stronger in the nuclei. In agreement with our data, the PSORT II program (http://psort.ims.u-tokyo.ac.jp) predicts that the DNA2 protein is a nuclear protein or a mitochondrial or a cytoplasmic protein. Our data suggest that the DNA2 protein participates in DNA damage repair and replication in nuclear and mitochondrial genomes.

A main goal in cancer research is to find mutations that are causally implicated in cancer pathogenesis. Alteration in DNA damage repair and replication has long been recognized as an important step in cancer development.\(^ {1,38}\) DNA2 mutations detected in the present study are novel somatic mutations in human cancers. Moreover, the frameshift mutations identified in this study would lead to premature stops of amino acid synthesis in the affected proteins and hence resembles a typical loss-of-function mutation. From an earlier observation that DNA2 depletion led to accumulation of aneuploid cells and internuclear bridges\(^ 5\) that may represent genomic instability, telomere instability and defective DNA repair, mutation of the DNA2 gene and loss of DNA2 protein expression might contribute to development of GCs and CRCs by altering cell cycle and/or DNA damage signaling/repair. It is imperative that additional functional studies on the mutated DNA2 gene and its product using in vitro models and animal models should be done. Also, it should be determined whether mutation and loss of expression of the DNA2 gene are common features of cancers. Mutations and expression status of the DNA2 gene should be further analyzed in other cancers.

**REFERENCES**

1. Hakem R. DNA-damage repair: the good, the bad, and the ugly.


