Inactivating mutations of the human base excision repair gene NEIL1 in gastric cancer

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Oxidized DNA base lesions, such as thymine glycol (Tg) and 8-hydroxyguanine, are often toxic and mutagenic and have been implicated in carcinogenesis. To clarify whether NEIL1 protein, which exhibits excision repair activity towards base lesions, is involved in gastric carcinogenesis, we examined 71 primary gastric cancers from Japanese patients and four gastric cancer cell lines for mutations and genetic polymorphisms of the NEIL1 gene. We also examined 20 blood samples from Chinese patients for NEIL1 genetic polymorphisms. Three mutations (c.82_84delGAG:p.Glu28del, c.936G>A and c.1000A>G:p.Arg334Gly) and two genetic polymorphisms were identified. When the excision repair activity towards double-stranded oligonucleotide containing a Tg:A base pair was compared among six types of recombinant NEIL1 proteins, p.Glu28del-type NEIL1, found in a primary case, was found to exhibit an extremely low activity level. Moreover, c.936G>A, located in the last nucleotide of exon 10 and detected in the KATO-III cell line, was shown to be associated with a splicing abnormality using an in vivo splicing assay. An immunofluorescence analysis showed that the wild-type NEIL1 protein, but not the truncated protein encoded by the abnormal transcript arising from the c.936G>A mutation, was localized in the nucleus, suggesting that the truncated protein is unlikely to be capable of repairing nuclear DNA. An expression analysis revealed that NEIL1 mRNA expression was reduced in six of 13 (46%) primary gastric cancer specimens that were examined. These results suggest that low NEIL1 activities arising from mutations and reduced expression may be involved in the pathogenesis in a subset of gastric cancers.

Materials and methods

Samples
Gastric cancers from a total of 71 sporadic cases and corresponding normal mucosae were obtained from Hamamatsu University Hospital, Shizuoka, Japan. The four gastric cancer cell lines used in this study were MKN45, MKN74, TMK-1 and KATO-III (30). Blood samples from a total of 20 Chinese primary gastric cancer patients were obtained from Huai'an City Municipal Hospital, China (31). The protocol was approved by the Human Institutional Review Board of Hamamatsu University School of Medicine. DNA was extracted by standard SDS-protease K digestion followed by phenol-chloroform extraction and ethanol precipitation.

NEIL1 variant screening
NEIL1 variants were screened with the WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE). The method is based on ion-pair, reverse-phase high-performance liquid chromatography and temperature-modulated heteroduplex analysis. Nine coding exons of the NEIL1 gene were amplified by genomic polymerase chain reaction (PCR) using 10 sets of primers: 5'–AGC CGC TAC CTC ACA AAG TC-3' and 5'–GCT GAA AAG

Abbreviations: FITC, fluorescein 5-isothiocyanate; oh8G, 8-hydroxyguanine; PAGE, polyacrylamide gel electrophoresis; PBGD, porphobilinogen deaminase; PCR, polymerase chain reaction; QRT–PCR, quantitative real-time-PCR; RT–PCR, reverse transcription–PCR; Tg, thymine glycol

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AGC CGG ACA TG-3' for exon 4a, 5'-CTA CGG CAT CTC AGC TG-3' and 5'-AGA AGG CAC TAA GAG AGT CAG-3' for exon 4b, 5'-ACG CAC CCA GAC GCT GTT C-3' and 5'-GGC AAG CCA TGC TAG GCA G-3' for exon 5, 5'-AGA GAT CCT GTA CCG GTC AG-3' and 5'-CCC CTG TGT TTG AGC GTA AA GC-3' for exon 6, 5'-AGC TCA GAG AGA AGT GCA G-3' and 5'-TGT AGA CTA CCG CTC GCT TG-3' for exon 7. 5'-TCA GTC CAT CAG CTT GTG TAG-3' and 5'-CTA GAC AGA CCC TCA GCA TC-3' for exon 8, 5'-CTC GCC TCT CAA AGG AAT AC-3' and 5'-TGT AGC CTT TGG AGT C-3' for exon 9, 5'-CCA ACT CCA ACA CCA GTG TG-3' and 5'-AGG GTA AGG CAG CTT CCT GCT G-3' for exon 10, 5'-ACC TCT GAA CGT TCT CTT GAG-3' and 5'-CTT TGT TCA CGC CTC CCT AG-3' for exon 11, 5'-CAC CAC GGT GTG CTT C-3' and 5'-ACC TTA GAG TAT CCT GTC CTC T-3' for exon 12. PCR was performed in 25 μl reaction mixtures containing AmpliTaq Gold (Applied Biosystems, Tokyo, Japan) under the following conditions: 30 s at 94°C, 30 s at 56°C and 60 s at 72°C for 35 cycles. PCR products were analyzed using WAVE and WAVEMaker software (4.0, Transgenomic). PCR products exhibiting different peaks in the WAVE analysis were directly sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI 3100 Genetic Analyzer (Applied Biosystems).

Preparation of the recombinant NEIL1 proteins

Preparation of the recombinant NEIL1 proteins was performed using a variety of methods. The NEIL1 protein tagged with His6 at its C-terminus was prepared according to the pET System protocol (Novagen, Darmstadt, Germany). Briefly, NEIL1 cDNA-containing pET28β(+) vector was inserted into a pET28b-vector NEIL1 cDNA vector. After the insertion, the recombinant plasmid was transformed into E. coli for protein expression. The recombinant E. coli cells were cultured in LB medium containing ampicillin (100 μg/ml) at 37°C until A600 reached 0.5. After incubation with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 25°C for 24 h, the cells were fixed with 4% paraformaldehyde. After microwave treatment and permeabilization, the cells were incubated with 40 μg/ml of anti-FLAG M2 antibody (Sigma, St Louis, MO) at room temperature for 1 h. Indirect immunofluorescence labeling was performed using a fluorescein 5-isothiocyanate (FITC)-conjugated anti-FLAG mAb as secondary antibody (Cappel, Aurora, OH) at room temperature for 30 min, and the nuclei were stained with 4'-6-diamidino-2-phenylindole (DAP) (Vysis, Downers Grove, IL). The slides were examined under a fluorescence microscope (Olympus BX-50-FL, Olympus) equipped with epifluorescence filters and aphotometric CCD camera (Quantix 1400; Roper Scientific, Tucson, AZ). The images captured were digitized and stored in the image analysis program (IPLab Spectrum; Scanalytics, Fairfax, VA).

Quantitative real-time (QRT)-PCR

Expression of the NEIL1 mRNA transcript was measured by QRT-PCR using a LightCycler instrument (Roche, Palo Alto, CA). The samples available for analysis included 10 normal gastric mucosa tissues and 20 gastric cancer tissues. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and subsequently treated with RNase-free DNase (Promega, Madison, WI) according to the supplier’s instructions. cDNA was synthesized using a QuantiTect SYBR Green PCR kit (Qiagen). The following PCR primers were used: 5'-AGA AGA TAA GGA CCA AGC TGC-3' and 5'-GAT CCC CCG GAG AGA TG-3' for the NEIL1 transcript and 5'-GTC TGG TAA CCG CAA TGG C-3' and 5'-TCC CTT GTG GTG GAC ACA C-3' for the GAPDH transcript. The relative amounts of NEIL1 transcript were standardized according to those of the GAPDH transcript. The relative expression of each sample was calculated by comparing the expression of non-cancerous gastric tissue in a primary gastric cancer patient (case no. 1). The T/N ratio was calculated in each case.

Identification of NEIL1 mutations in gastric cancer

We examined 71 primary gastric cancers from Japanese patients and four gastric cancer cell lines for mutations and genetic polymorphisms of the NEIL1 gene. We also examined blood samples from 20 Chinese primary gastric cancer patients for genetic polymorphisms of the NEIL1 gene. A survey for all the coding sequences of the NEIL1 gene with intronic or non-coding sequence-based primers disclosed three mutations (Table I). None of the three were observed in any of the non-cancerous tissues examined in this study, and all of the mutations were observed in heterozygosity.

Impairment in Tg DNA glycosylase activity of mutant-type NEIL1 protein

To assess the functional status of the proteins coded by the variant-type NEIL1 genes, each type of recombinant NEIL1 protein tagged with His6 at its C-terminus was prepared by site-directed mutagenesis in a wild-type NEIL1 background. The mutant-type NEIL1 proteins were expressed as fusion proteins with 6xHis at their C-terminus in E. coli DH5α. The recombinant NEIL1 proteins were purified using Ni2+ affinity chromatography. The purified proteins were tested for DNA glycosylase activity using an in vitro assay system.
cDNA-containing pET25b(+) expression vector. Since the N-terminal Pro2 is an active site (19), we also prepared a p.Pro2del construct as a negative control. Although a p.Lys242Arg variant was not detected in the present series, this variant and p.Lys245Arg variant described here is available on the LocusLink homepage of the NCBI web site. Thus, we also prepared a p.Lys242Arg construct. In total, six recombinant NEIL1-His6 proteins, including a wild-type protein, were expressed in E.coli and purified to apparent homogeneity (Figure 1A). Their molecular size of ~47 kDa was determined by SDS–PAGE and corresponded with that calculated from the cDNA sequences. The Tg DNA glycosylase activity of the NEIL1 protein was tested by determining its capacity to cleave a double-stranded oligonucleotide containing a Tg:A mispair (Figure 1B and C). The enzymatic activities of the p.Lys242Arg, p.Gly245Arg and p.Arg334Gly type proteins were similar to that of the wild-type protein. However, the enzymatic activity of the p.Glu28del type protein was similar to that of the p.Pro2del type protein, indicating a strong impairment in the DNA repair activity of the p.Glu28del type protein.

**Effect of the NEIL1 c.936G>A mutation on pre-mRNA splicing and subcellular localization**

c.936G>A was detected in one gastric cancer cell line, KATO-III. As this nucleotide substitution has not been found in any other samples, it is probably a somatic mutation. However, as DNA from corresponding normal tissue for the KATO-III cell line was not available, we could not exclude the possibility that this substitution might be a rare genetic polymorphism. c.936G>A was located in the last nucleotide of exon 10. Guanine at the last nucleotide of the exon resides in the consensus sequence at the 5’ splice site, which is recognized and bound by U1 small nuclear RNP during 5’ splice site selection (32). Moreover, the G>A change at the last nucleotide of the exon in the OGG1 gene was associated with the retention of the following intron (13). Therefore, the c.936G>A change in the NEIL1 gene may weaken the splicing signal. RT–PCR and sequencing analyses revealed that a NEIL1 mRNA transcript derived from KATO-III cells, but not from TMK-1 cells, contained a 160-bp intron 10 sequence (Figure 2A, band II). The transcript derived from KATO-III cells encoded the truncated-type NEIL1 protein (Figure 2B). To confirm the contribution of c.936G>A to this splicing abnormality, a pALTER-MAX mammalian expression vector was inserted by a PCR product containing c.936G or c.936A and surrounding the NEIL1 sequence, including intron 10, as illustrated in Figure 2C. These vectors and parental pALTER-MAX vector were then transfected into NCI-H1299 cells, and the total RNA was extracted from these cells. An RT–PCR analysis using a set of primers for the pALTER-MAX sequence and a sequencing analysis of the bands on the gel revealed that a transcript derived from the cells transfected with the NEIL1-c.936A type vector, but not from those with the NEIL1-c.936G type vector, contained the 160-bp intron 10 sequence (Figure 2D). These results indicate that the c.936G>A change caused the splicing abnormality. This abnormal splicing pattern leads to the replacement of the 78 aa C-terminus with 25 unrelated amino acids, resulting in the deletion of part of the nuclear localization signal (NLS). As the NEIL1 protein has been reported to be localized in the cell nucleus (20,21), we investigated whether the structural change resulting from the c.936G>A mutation actually affected subcellular localization. Wild-type and truncated-type NEIL1 cDNA fused with the FLAG sequence at the C-terminus were inserted into pcDNA3.1 mammalian expression vectors, and then transiently transfected into the NCI-H1299 cell line. The presence of exogenous NEIL1-FLAG proteins in the transfected cells was then examined using fluorescence microscopy (Figure 3). Consistent with previous results (20,21), the wild-type NEIL1-FLAG protein was localized in the nucleus; however, the truncated-type NEIL1-FLAG protein was localized in the cytoplasm. We also evaluated the subcellular localization of p.Arg334Gly-type protein in the same experimental design, however, the protein was localized in the nucleus (Figure 3). These results indicate that the c.936G>A-type truncated NEIL1 protein, lacking an NLS, is localized in the cytoplasm, unlike the nuclear localization of the wild-type NEIL1 protein, suggesting that the excisional repair ability of the truncated-type NEIL1 protein is impaired, preventing oxidatively damaged bases in nuclear DNA from being corrected.

**Reduction in NEIL1 expression in gastric cancer**

We next assessed the levels of NEIL1 mRNA expression in 13 primary gastric cancers and corresponding non-cancerous mucosae by QRT–PCR using the PBGD housekeeping gene as a control. The ratio of the NEIL1 mRNA expression level in the tumor compared with the corresponding non-cancerous tissue (T/N ratio) was calculated in each case (Figure 4). Six out of 13 (46%) of the primary gastric cancers exhibited a

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**Table I. Somatic mutations and genetic polymorphisms of the NEIL1 gene**

<table>
<thead>
<tr>
<th>Exon/intron</th>
<th>Nucleotide change&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Predicted effect (polypeptide length&lt;sup&gt;c&lt;/sup&gt;)</th>
<th>Sample</th>
<th>Allele frequency&lt;sup&gt;d&lt;/sup&gt;</th>
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<td><strong>Somatic mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Exon 4</td>
<td>c.82_84delGAG</td>
<td>p.Glu28del (389 aa)</td>
<td>GC-16 (surgical specimen)</td>
<td>0.000</td>
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<tr>
<td>Exon 10</td>
<td>c.936G&gt;A</td>
<td>splicing abnormality (337 aa)</td>
<td>KATO-III (cell line)</td>
<td>0.000</td>
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<tr>
<td>Exon 11</td>
<td>c.1000A&gt;G</td>
<td>p.Arg334Gly (390 aa)</td>
<td>GC-71 (surgical specimen)</td>
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<tr>
<td><strong>Genetic polymorphism</strong></td>
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<tr>
<td>Intron 7</td>
<td>[c.719-80delT + c.719-82T&gt;G]</td>
<td>Unknown</td>
<td>JS-159 (blood sample)</td>
<td>0.000</td>
</tr>
<tr>
<td>Exon 8</td>
<td>c.733G&gt;A</td>
<td>p.Gly245Arg (390 aa)</td>
<td>JS-158 (blood sample)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

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<sup>a</sup> According to LocusLink homepage (Locus ID: 79661) of the NCBI web site.

<sup>b</sup>Nucleotide +1 is the A of the ATG-translation initiation codon.

<sup>c</sup>Length of wild-type NEIL1 protein: 390 aa.

<sup>d</sup>The allele frequencies were determined using analyses of non-cancerous tissues from Japanese and Chinese patients with gastric cancer.

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Fig. 1. Comparison of the cleavage activity toward double-stranded DNA containing a Tg:A mispair among NEIL1 variant proteins. (A) Purification of recombinant NEIL1 proteins resolved by PAGE and stained with Coomassie Brilliant Blue. NEIL1 protein tagged with His6 at its C-terminus was prepared according to the pET System protocol. Lysates of E.coli culture with/without expression of wild-type (wt) NEIL1 protein (lanes 2–5) and purified various NEIL1 proteins (lanes 6–11) are shown. Pa. (lanes 2 and 3) and wt (lanes 4 and 5) indicate the transformation of the host cells with parental pET25b(+) and NEIL1 cDNA-containing pET25b(+) vector, respectively. -- and + mean the absence and presence of IPTG induction, respectively. The NEIL1-His6 protein band is indicated. (B) Cleavage activity of NEIL1 variant proteins toward double-stranded DNA containing a Tg:A mispair. Various types of NEIL1 proteins and a 32P-labeled double-stranded oligonucleotide containing or not containing a single 5'R,6'S-Tg:A mispair were incubated and subjected to 20% PAGE. A 32P-labeled 13mer oligonucleotide was used as a size marker for the cleaved products. A representative result is shown in the panel. (C) The percentage of cleaved products per total under conditions of 37°C for 15 min was calculated as % incision and listed.

<table>
<thead>
<tr>
<th>NEIL1 protein</th>
<th>% incision (average ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>33.4 ± 1.01</td>
</tr>
<tr>
<td>Pro2del</td>
<td>4.6 ± 0.16</td>
</tr>
<tr>
<td>Glu28del</td>
<td>7.7 ± 0.10</td>
</tr>
<tr>
<td>Lys242Arg</td>
<td>30.2 ± 4.42</td>
</tr>
<tr>
<td>Gly245Arg</td>
<td>32.8 ± 0.23</td>
</tr>
<tr>
<td>Arg334Gly</td>
<td>32.5 ± 1.21</td>
</tr>
</tbody>
</table>
A change by in vivo splicing assay. (A) Detection of the abnormally spliced product from KATO-III cell line by RT–PCR analysis. The genotypes of TMK-1 and KATO-III cells are homozygous for the c.936G allele and heterozygous for the c.936G and c.936A alleles, respectively. Expression of the NEIL1 mRNA transcript was examined using the primers located in exons 10 and 11. GAPDH expression was also examined. The band detected by electrophoresis was excised from the gel and directly sequenced. Bands indicated by arrowheads I and II are PCR products retaining or not retaining intron 10, respectively. (B) Diagram showing the polypeptide sequence translated from the read-through transcript of intron 10. (C) Schema of a part of pALTER-MAX expression vectors containing exon 10, intron 10 and exon 11 of the NEIL1 gene. (D) In vivo splicing assay. The NEIL1-c.936G and -c.936A type expression vectors were transiently transfected into the NCI-H1299 cell line. At 24 h post-transfection, the cells were harvested, and RT–PCR was performed using the set of primers pAM-F/pAM-R, as indicated in (C), or a set of primers for GAPDH. PCR products were fractionated by agarose gel electrophoresis, and each band was directly sequenced. The 119-, 260- and 420-bp products correspond to a vector sequence without intron 10, and a NEIL1 sequence with intron 10, respectively.

Fig. 2. Detection of the NEIL1 splicing abnormality due to the c.936G > A change in in vivo splicing assay. (A) Detection of the abnormally spliced product from KATO-III cell line by RT–PCR analysis. The genotypes of TMK-1 and KATO-III cells are homozygous for the c.936G allele and heterozygous for the c.936G and c.936A alleles, respectively. Expression of the NEIL1 mRNA transcript was examined using the primers located in exons 10 and 11. GAPDH expression was also examined. The band detected by electrophoresis was excised from the gel and directly sequenced. Bands indicated by arrowheads I and II are PCR products retaining or not retaining intron 10, respectively. (B) Diagram showing the polypeptide sequence translated from the read-through transcript of intron 10. (C) Schema of a part of pALTER-MAX expression vectors containing exon 10, intron 10 and exon 11 of the NEIL1 gene. (D) In vivo splicing assay. The NEIL1-c.936G and -c.936A type expression vectors were transiently transfected into the NCI-H1299 cell line. At 24 h post-transfection, the cells were harvested, and RT–PCR was performed using the set of primers pAM-F/pAM-R, as indicated in (C), or a set of primers for GAPDH. PCR products were fractionated by agarose gel electrophoresis, and each band was directly sequenced. The 119-, 260- and 420-bp products correspond to a vector sequence without NEIL1, a NEIL1 sequence without intron 10, and a NEIL1 sequence with intron 10, respectively.

Discussion

Three mutations and two genetic polymorphisms in the NEIL1 gene locus were identified in the present study. When the Tg DNA glycosylase activity was compared among six types of recombinant NEIL1 proteins, p.Glu28del-type NEIL1, first identified in a primary gastric cancer, was found to exhibit an extremely low activity level. Moreover, the c.936G > A truncated NEIL1 protein, first detected in the KATO-III cell line, was shown to be associated with a splicing abnormality using an in vivo splicing assay. In contrast to the results for the wild-type NEIL1 protein, an immunofluorescence analysis showed that the c.936G > A-type truncated NEIL1 protein was localized in the cytoplasm, not the nucleus, suggesting that the truncated protein’s ability to correct nuclear DNA damage via its excision repair ability was likely to be impaired. An expression analysis revealed that NEIL1 mRNA expression was reduced in six of 13 (46%) of the primary gastric cancer specimens that were examined. These results suggest that low NEIL1 activities arising from mutations and reductions in expression may be involved in the pathogenesis of a subset of gastric cancers.

In this study, the NEIL1-p.Glu28del-type protein resulting from the c.82_84delGAG mutation was shown to exhibit a greatly reduced ability to cleave a double-stranded oligonucleotide containing a single 5R,6S-Tg:A mispair. As mammalian NEIL1 protein has been shown to efficiently excise the substrate (23,26), the assay system used in this study was expected to be adequate to determine the level of the NEIL1 protein’s repair ability. As expected, the repair ability of the p.Glu28del-type protein as well as the p.Pro2del-type protein, which is known to be an inactive form, was lower than that of the wild-type protein. However, the reason for the reduction in the repair ability was unclear, because the Glu28 residue is not located in a domain that is known to be necessary for the protein’s repair function. The Glu28 residue may play an important role in the recognition of the DNA substrate, or some other related function. In the future, a crystal structure analysis of the NEIL1 protein alone and covalently complexed with DNA, in conjunction with the present finding that the p.Glu28del-type protein exhibited an extremely low activity level, would contribute to establishing further correlations between the structure and repair function of the NEIL1 protein.

The c.936G > A mutation caused a splicing abnormality that led to the production of an aberrant mRNA transcript encoding...
a truncated NEIL1 protein. Unlike the wild-type protein, the truncated NEIL1 protein was not localized in the nucleus, suggesting that the c.936G>A-type truncated protein could not repair nuclear DNA. KATO-III cells carrying this c.936G>A mutation also showed a reduction in NEIL1 mRNA expression, agreeing with the observation that NEIL1 mRNA expression was reduced in some primary gastric cancers in the present study. Thus, both genetic alterations may contribute to the low NEIL1 activity in the KATO-III cell line.

Regarding the prevalence of somatic mutations in human gastrointestinal cancer, Wang et al. (33) reported that only three non-synonymous somatic mutations, each in a different gene, were identified in ~3.2 Mb of coding colon cancer DNA, containing 1811 exons from 470 genes. Considering this prevalence, the observation of three NEIL1 mutations in this study implies a relatively high prevalence of NEIL1 somatic mutations in gastric cancer. Gastric cells with a NEIL1 somatic mutation may have been selected during tumorigenesis. However, as we did not compare the prevalence of the NEIL1 mutation in a variety of human cancers, it is unknown whether its prevalence in gastric cancer is significantly higher than in other cancers. We are planning to investigate this issue in the near future.

A reduction in the DNA repair function of the NEIL1 protein in human cells, particularly, where severe oxidative stress is present, could lead to a high level of accumulated oxidative DNA damage. This situation might lead to an increase in the incidence of gene mutation in the cells. Moreover, a 20--35% reduction in the repair capacity of cells has been associated with a 4--6-fold higher risk of developing cancer (34). Thus, it is suggested that low NEIL1 activities caused by mutations and reduced mRNA expression may contribute to the alteration of genes involved in gastric carcinogenesis in a subset of gastric cancers.
Acknowledgements

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