Tissue factor pathway inhibitor 2 (TFPI2) is frequently silenced by aberrant promoter hypermethylation in gastric cancer

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Abstract
Aberrant methylation of promoter CpG islands is associated with transcriptional inactivation of tumor-suppressor genes in cancer. TFPI2, a Kunitz-type serine proteinase inhibitor, has been identified as a putative tumor-suppressor gene from genome-wide screening for aberrant methylation, using a microarray combined with the methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-aza-dCyd) in various types of tumors. We assessed the methylation status of TFPI2 and investigated its expression pattern in human primary gastric cancer (GC) tissues and in GC cell lines. Hypermethylation of the promoter CpG island, which was observed in more or less all of GC cell lines, was prevalent in a high proportion of primary GC tissues (15/18, or 83%), compared with noncancerous (4/18, or 22%) or normal (0/3, or 0%) stomach tissues, and expression of TFPI2 mRNA was reduced in 7 of the 17 primary GC tissues (41%). Moreover, immunohistochemical analyses showed decreased levels of TFPI-2 protein, compared with adjacent noncancerous tissues in 8 of the 20 primary GC tissues examined (40%). TFPI2 mRNA expression was restored in gene-silenced GC cells after treatment with 5-aza-dCyd. Aberrant methylation of TFPI2 promoter CpG island occurred not only in GC cells but also in primary GC tissues at a high frequency, suggesting that epigenetic silencing of TFPI2 may contribute to gastric carcinogenesis. © 2010 Elsevier Inc. All rights reserved.

1. Introduction
CpG islands are short CpG-rich regions, often mapping to the promoter and exonic regions of the gene [1]. Increased methylation of CpG islands within gene promoters is associated with transcriptional silencing of the gene [2], and hypermethylation of CpG islands in the promoter region of tumor-suppressor genes (TSGs) is a well-known mechanism of gene silencing that contributes to the development and progression of cancer [3]. Aberrant promoter methylation of TSGs or candidate TSGs that may play crucial roles in tumorigenesis, such as CDKN2A (alias p16) [4,5], CDH1 [6], MLH1 [4], RUNX3 [7], DLC1 [8], ADAM23 [9], and SFRP genes [10], has been reported in gastric cancer (GC), as well as in other human cancers.

Recently, various techniques have been developed to efficiently and effectively identify genes with epigenetic aberrations [11]. One such technique screens genes treated with a demethylating agent, the methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-aza-dCyd), using a microarray, to pick up candidate methylation-regulated genes genome-wide [12]. With this microarray technique, the tissue factor pathway inhibitor 2 gene (TFPI2) was identified in pancreatic ductal adenocarcinoma [13], malignant melanoma [14], and hepatocellular carcinoma [15], and GC [16].

Further studies on the effect of epigenetic inactivation of the TFPI2 gene on tumorigenesis, including proliferation and invasion, implicate it as a putative TSG. TFPI-2 expression was demonstrated immunohistochemically in neoplastic cells of laryngeal, breast, gastric, colon, pancreatic, renal, endometrial cancer and glial neoplasms, as well as normal tissue of the breast, stomach, colon and pancreas [17]. Normal gastric tissue also expressed TFPI-2; however, the intensity of staining was not uniform, with higher intensity in more differentiated tumors, suggesting that the expression of TFPI-2 might diminish with an increasing degree of malignancy.

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In the present study, we investigated the expression and methylation status of the \textit{TFPI2} gene in human GC tissues and cell lines and evaluated whether hypermethylation of its promoter CpG island might be involved in gene silencing and therefore contribute to gastric carcinogenesis.

2. Materials and methods

2.1. Cell lines and primary tumors

Nine human GC cell lines were used: MKN1, MKN28, MKN45, MKN74 [18,19], TPK1 [20], NUGC3 [21], SNU16 [22], KATO-III [23], and AZ521 [24]. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/mL penicillin—100 µg/mL streptomycin.

Primary tumor samples were obtained from 20 patients who were undergoing surgery at the University Hospital of Kyoto Prefectural University of Medicine. Surgical specimens were fixed in formalin and embedded in paraffin. From 18 other patients and 3 healthy male volunteers, we obtained paired samples from cancerous and noncancerous tissues and normal stomach tissues, respectively, at the time of upper gastrointestinal endoscopy. All biopsy specimens were frozen immediately in liquid nitrogen and stored at −70°C until needed. Genomic DNA and total RNA were isolated from all cell lines and primary tumors using a DNeasy tissue kit, RNeasy mini kit, and AllPrep DNA/RNA mini kit (Qiagen, Valencia, CA; Tokyo, Japan). Informed consent and Ethics Committee approval were obtained before initiation of the study.

2.2. Reverse transcription-polymerase chain reaction

For reverse transcriptase–polymerase chain reaction (RT-PCR), single-stranded cDNAs were generated from total RNAs using QuantiTect reverse transcription kits according to the manufacturer’s protocol (Qiagen), and were amplified with primers specific for \textit{TFPI2}. The \textit{GAPDH} gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was amplified concurrently to estimate the efficiency of cDNA synthesis. Quantitative real-time PCR experiments were performed with a LightCycler system using FastStart DNA Master Plus SYBR Green I according to the manufacturer’s protocol (Roche Diagnostics, Penzberg, Germany). Primer sequences and PCR conditions are given in Table 1.

2.3. Combined bisulfite restriction analysis and bisulfite sequencing

Genomic DNA was treated with sodium bisulfite using an EZ DNA methylation kit (Zymo Research, Orange, CA), and amplified by PCR using primer sets designed to amplify the region harboring CpG-1 (Table 1). For combined bisulfite restriction analysis (COBRA), PCR products were digested with the \textit{BstUI} restriction enzyme, which recognizes sequences unique to the methylated alleles but cannot recognize unmethylated alleles, and were examined by electrophoresis [25]. DNA from peripheral blood lymphocytes of a healthy male volunteer served as a negative control for COBRA assays, and CpGenome universal methylated DNA (Millipore—Chemicon, Billerica, MA) served as a positive control for methylated alleles. For bisulfite sequencing, PCR products were subcloned and then sequenced.

2.4. Immunohistochemistry

Indirect immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections, as described elsewhere [24]. The sections were dewaxed and rehydrated in graded concentrations of ethanol. Antigens were retrieved by microwave pretreatment in 10 mmol/L citrate buffer (pH 6.0) for 20 minutes at 95°C. After cooling, the sections were treated with 3% hydrogen peroxide to block endogenous peroxidase, and then reacted overnight at 4°C with a rabbit polyclonal antibody against \textit{TFPI2} (1:100, H-120; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were rinsed, incubated with a second antibody (Histfine simple stain MAX-PO [MULTI]; Nichirei, Tokyo, Japan) at room temperature for 20 minutes, stained with 0.6% hydrogen peroxide and 3,3′-diaminobenzidine (Histfine; Nichirei), and counterstained with hematoxylin.

2.5. Drug treatment

Cells were treated with 1 or 5 µmol/L of 5-aza-dCyd for 4 days with or without the addition of 50 ng/mL trichostatin A (TSA), a histone deacetylase inhibitor, for 1 day. For 5-aza-dCyd treatment, GC cells were seeded on day 0, treated with 1 or 5 µmol/L of 5-aza-dCyd (Methylation Ltd., Port Orange, FL) for 48 hours on days 1 and 3, and harvested on day 5, as described elsewhere [14]. For the synergistic study, cultures were incubated with 1 or 5 µmol/L of 5-aza-dCyd for 4 days, following the addition of 0 or 50 ng/mL TSA during the last 24 hours of incubation.

3. Results

3.1. Expression of \textit{TFPI2} mRNA in GC cell lines

Expression of \textit{TFPI2} mRNA was analyzed in human GC cell lines by RT-PCR (Fig. 1A). Of the nine GC cell lines, all except AZ521 completely lacked expression of \textit{TFPI2}, and the AZ521 cell line exhibited an apparently reduced expression, compared with the normal stomach tissue. Having confirmed the expression status of \textit{TFPI2} in these cell lines, we next examined the promoter methylation status of this gene.
3.2. Methylation status of the TFPI2 CpG island in GC cell lines

Hypermethylation in the CpG-rich promoter or exonic regions is strongly associated with transcriptional silencing of TSGs. First, we identified a 275-bp sequence (−171 to +104, CpG-1) (Fig. 1B) as a CpG island around exon 1 of TFPI2 by means of the European Molecular Biology Laboratory—European Bioinformatics Institute (EMBL—EBI) genome database and software (http://www.ebi.ac.uk/emboss/cpgplot/).

To explore the correlation between gene silencing and CpG promoter methylation, we assessed the methylation status of CpG-1 in the nine GC-derived cell lines with or without expression of this gene, by means of COBRA with the BssUI restriction enzyme for each of the PCR fragments. The GC cells lacking TFPI2 expression were found to be aberrantly hypermethylated, except for MKN45 cells, which were partly unmethylated. In contrast, AZ521 cells, which had a decreased level of TFPI2 mRNA, were relatively hypomethylated (Fig. 1C).

Table 1

<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Ta, °C</th>
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<tr>
<td>RT-PCR</td>
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<tr>
<td>TFPI2</td>
<td>5'-GCCATGTCGTCTCAGGAG</td>
<td>5'-TCTGGGTACCTGTGAGTAG</td>
<td>67</td>
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<tr>
<td>GAPDH</td>
<td>5'-CGGAGTCACGGATGTCTCGAAT</td>
<td>5'-AGCCCTTCCCATGGTGAGAC</td>
<td>67</td>
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<tr>
<td>COBRA and bisulfite sequencing</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TFPI2</td>
<td>5'-AGGTAGGTTTAAATTGTTTGG</td>
<td>5'-CTATTACTCTAAAACACATC</td>
<td>58</td>
</tr>
</tbody>
</table>

Abbreviations: COBRA, combined bisulfite restriction analysis; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase—polymerase chain reaction; Ta, annealing temperature.

Fig. 1. (A) Expression of TFPI2 in nine gastric cancer (GC) cell lines and normal stomach, detected by reverse transcriptase—polymerase chain reaction (RT-PCR) analysis. Note that eight of the nine cell lines (89%) showed almost a complete silencing of this gene and the remaining cell line (11%) showed an apparently decreased expression, compared with normal stomach. DW, distilled water. (B) Schematic map of a CpG island around exon 1 of the TFPI2 gene identified using EMBL—EBI genome database and software (http://www.ebi.ac.uk/emboss/cpgplot/). Exon 1 is indicated by an open box, and the transcription start site is marked at +1. CpG sites are indicated by vertical ticks on the expanded axis. CpG-rich regions around exon 1 (CpG-1) were examined in the bisulfite-PCR analysis and bisulfite sequencing. For the bisulfite-PCR analysis, arrowheads indicate restriction sites for BssUI. (C) Result of bisulfite-PCR analysis of TFPI2 CpG-1 in GC cell lines after digestion with BssUI. Arrows and arrowheads indicate digested fragments (methylated DNA: M) and undigested products (unmethylated DNA: U), respectively. PLC, normal peripheral lymphocytes; M-DNA, CpGenome universal methylated DNA. (D) Results of bisulfite sequencing of the TFPI2 CpG-1, examined in cell lines MKN1, MKN28, and MKN45, which lost expression of TFPI2 mRNA. All 29 of the CpG sites were sequenced. Each square indicates CpG dinucleotides: open squares, unmethylated; solid squares, methylated. Percentages indicate the fraction of methylated CpG dinucleotides.
Further analysis by bisulfite sequencing of the methylation status of each CpG dinucleotide for TFPI2 CpG-1 showed that CpG sites on the CpG-1 tended to be extensively methylated in TFPI2-nonexpressing cell lines (MKN1 and MKN28) and partial hypomethylated in the cell line MKN45, which is known to contain a few hypomethylated alleles (Fig. 1D).

3.3. Aberrant DNA methylation of TFPI2 in primary GC tissues and correlation with its mRNA expression

Because hypermethylation of the promoter region around exon 1 would likely be associated with silencing of TFPI2 expression in GC cells, we next assessed the methylation status of TFPI2 CpG-1 in primary GC tissues by COBRA. For methylation analysis, paired GC tissues

Fig. 2. (A) Representative results of bisulfite-PCR analyses of TFPI2 CpG-1 after digestion with BstUI in 18 primary paired GC tissues and three normal stomach tissues. The arrows and arrowheads indicate digested fragments (methylated DNA: M) and undigested products (unmethylated DNA: U), respectively. (B) Methylation status of TFPI2 CpG-1 was determined by bisulfite sequencing in two representative paired GC tissue samples. N, noncancerous tissue; T, GC tissue; NS, normal stomach tissue. Percentages indicate the fraction of methylated CpG dinucleotides. (C) Expression of TFPI2 mRNA in the same primary cases of GC tissues and the paired noncancerous tissues. In cases 2, 3, 4, 6, 8, 12, and 15, in which tumors had aberrant hypermethylation of TFPI2 CpG-1 (panel B), tumors showed only traces of TFPI2 expression, compared with the corresponding noncancerous tissues. NA, not available.
and noncancerous gastric epithelial tissues were obtained during upper gastrointestinal endoscopic inspection from 18 patients who underwent biopsy. Methylation of TFPI2 CpG-1, which was observed in 4 of the 18 noncancerous stomach tissues (22%) and in none of three normal stomach tissues (0%), was detected in 15 of the 18 primary GC tissues (83%); in 10 of these 15 cases, TFPI2 CpG-1 was not methylated in the corresponding noncancerous stomach tissues (Fig. 2A).

To validate the COBRA results, we performed bisulfite sequencing of CpG-1 in individual alleles from representative GC samples and their corresponding noncancerous tissues. Bisulfite sequencing of CpG-1 in cases 4 and 6 revealed aberrant hypermethylation in GC tissues (although hypomethylated alleles were also present, probably because of normal tissue components included in tumor tissue), whereas the corresponding noncancerous tissues showed hypomethylation of this region (Fig. 2B). These findings indicate that methylation of TFPI2 CpG-1 does not arise during the passage of GC cell lines in vitro, but rather may be a true cancer-related event during the pathogenesis of GC. Moreover, the results of RT-PCR using the same primary paired samples demonstrated that 7 of the 15 GC tissues with aberrant methylation of CpG-1 (cases 2, 3, 4, 6, 8, 12, and 15) exhibited apparently lower TFPI2 expression than the corresponding noncancerous tissue (Fig. 2C).

3.4. Expression pattern of TFPI-2 protein in primary GC tissues

Because the limited size of biopsy samples prevented measurement of protein expression levels, we next performed immunohistochemical analysis of the TFPI-2 protein on 20 additional primary GC tissues. For immunohistochemistry, primary GC samples were obtained from 20 patients who underwent surgery. Gastric cancer is classified into two major histological types according to Lauren’s classification: intestinal type and diffuse type [26]. Table 2 summarizes the expression pattern and relationships with clinicopathological features of TFPI-2 in primary GC tissues. Representative immunostaining patterns of TFPI-2 are shown in Figure 3.

Of the 20 primary GC tissues, 8 (40%) lacked or had reduced TFPI-2 expression, compared with adjacent noncancerous tissues. No TFPI-2 expression was seen in the three tissue samples with signet ring-cell carcinomas. We found no significant correlation between TFPI-2 expression pattern and clinicopathological features (i.e., age, sex, histological type, staging, and metastasis to the lymph nodes).

3.5. Effect of demethylation by 5-aza-dCyd on TFPI2 expression

Aberrant methylation of DNA in 5’ regulatory regions harboring a greater than expected number of CpG dinucleotides is a key mechanism by which genes relevant to cancer initiation and progression can be silenced [27]. We found that the genomic sequence of TFPI2 harbors CpG-rich regions (CpG islands) around exon 1 (Fig. 1B). To investigate whether demethylation could restore the expression of TFPI2 mRNA, we treated GC cells lacking TFPI2 expression (MKN1, MKN28, MKN45 and TMK1) with 1 or 5 μmol/L of 5-aza-dCyd, a methyltransferase inhibitor, as previously described [14]. The expression of TFPI2 mRNA was restored in all four representative cell lines after treatment with 5-aza-dCyd in a dose-dependent manner (Fig. 4A). In addition, we observed elevated expression of mRNA in MKN28 cells after treatment with the histone deacetylase inhibitor TSA alone and enhancement of expression by 5-aza-dCyd treated with TSA in all GC cells, indicating that histone deacetylation might be also involved in the transcriptional silencing of TFPI2 for methylated GC cells. COBRA revealed that drug treatment caused effective gene demethylation (Fig. 4B).

Table 2

<table>
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<th>Characteristics</th>
<th>Cases, no.</th>
<th>Expression of TFPI-2 protein, no. (%)</th>
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<tbody>
<tr>
<td>Sample size</td>
<td>n = 20</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤70</td>
<td>8</td>
<td>N &gt; T 4 (50) 4 (50)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>12</td>
<td>N &gt; T 4 (33) 8 (67)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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<tr>
<td>Male</td>
<td>12</td>
<td>N &gt; T 4 (33) 8 (67)</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>N &gt; T 4 (50) 4 (50)</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
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<tr>
<td>Intestinal type</td>
<td>10</td>
<td>N &gt; T 3 (30) 7 (70)</td>
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<tr>
<td>Diffuse type</td>
<td>10</td>
<td>N &gt; T 5 (50) 5 (50)</td>
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<tr>
<td>Stage, TNM</td>
<td></td>
<td></td>
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<tr>
<td>Stage I + II</td>
<td>12</td>
<td>N &gt; T 6 (50) 6 (50)</td>
</tr>
<tr>
<td>Stage III + IV</td>
<td>8</td>
<td>N &gt; T 2 (25) 6 (75)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>11</td>
<td>N &gt; T 3 (27) 8 (73)</td>
</tr>
</tbody>
</table>

Abbreviations: N, noncancerous tissue; T, tumor tissue (gastric cancer); TNM, tumor—node—metastasis.

4. Discussion

TFPI-2, a structural homolog of TFPI, is a 32-kDa Kunitz-type serine proteinase inhibitor that inhibits plasmin, trypsin, chymotrypsin, cathepsin G, and plasma kallikrein [28–31]. The human TFPI2 gene is located at 7q22 [32] and is expressed abundantly in the full-term placenta and widely in a variety of adult human tissues, such as liver, skeletal, muscle, heart, kidney, and pancreas [33]. The long arm of chromosome 7 is frequently associated with genetic alterations in various human malignancies, including GC. Loss of heterozygosity on chromosome arm 7q is often observed in primary GC.
tissues, suggesting the presence of a potential TSG at 7q involved in the progression of GC [34–36].

Aberrant methylation of TFPI2 was observed in 102 of 140 pancreatic cancer xenografts and primary pancreatic adenocarcinomas (73%), and was more likely in older patients with pancreatic cancer [13]. Aberrant methylation of TFPI2 in endoscopically aspirated pure pancreatic juice was seen in 21 of 36 pancreatic cancer patients (58%), in 3 of 17 intraductal papillary mucinous neoplasm patients (18%), in 1 of 21 chronic pancreatitis patients (5%), and in 6 of 6 pancreatic cancer patients with liver metastasis (100%). Moreover, of 30 patients in stage IVa and IVb, 26 (87%) exhibited aberrant methylation in pure pancreatic juice [37]. In other studies, TFPI2 promoter methylation was detected in 16 of 34 hepatocellular carcinoma cases (47%) [15], in 7 of 7 esophageal adenocarcinoma cases (100%) [38], and in 5 of 17 metastatic melanoma specimens (29%) but not in any primary melanoma tissues [14]. Hypermethylation of the TFPI2 gene promoter was demonstrated in 12 of 40 of NSCLC cases (30%) and was more frequently associated with those of stages III or IV and lymph node metastasis [39]. Most recently, Jee et al. [16] reported that aberrant methylation for TFPI2 was detected in 123 of 152 primary GC cases (80.9%), and the survival of patients with methylated alleles was poorer than that of patients without methylated alleles.

In agreement with these earlier studies, most of the primary GC tissues (15/18, or 83%) as well as the nine GC cell lines examined in the present study showed aberrant methylation of the TFPI2 CpG island by COBRA. Large amounts of fragments restricted in the sites recognized as methylated CpG dinucleotides were more clearly detected in cancer specimens and were considered to have cancerspecific hypermethylation, although a few cases showed less
showed promoter methylation of the gastric carcinogenesis. In this study, most of primary GCs reported by Jee et al. [16]. These findings are concordant with those from the recent 40% of GCs lacked or reduced expression of TFPI-2 protein. Some experimental functional assays have been performed previously that assessed the effect of restoration of TFPI-2 expression on inhibition of tumor growth and invasion. Restored expression of TFPI-2 in nonexpressing tumor cells resulted in marked suppression in their proliferation, migration, and invasive potential in vitro in pancreatic cancer and hepatocellular carcinoma [13,15]. Other studies on stably TFPI-2-transfected prostate cancer and glioma cell lines also demonstrated the suppressive role of TFPI-2 on tumor invasive behavior [41,42]. Recently, it has been reported that TFPI-2 may have tumor suppressive potential for inducing apoptosis and inhibiting angiogenesis. Restoration of TFPI-2 activates both intrinsic and extrinsic caspase-mediated, proapoptotic signaling pathways and induces apoptosis in the human glioblastoma cell line [43]. Endogenous overexpression of TFPI-2 or addition of excess TFPI-2 effectively inhibits angiogenesis in vivo, as well as endothelial cell migration and capillary tube formation in vitro [44]. These findings support that idea that TFPI-2 acts as a tumor suppressor and that epigenetic silencing by aberrant hypermethylation of the promoter of the TFPI2 gene seems to be involved in the development of GC.

References


Fig. 4. (A) Representative results of quantitative real-time PCR analysis to reveal TFPI2 expression in MKN1, MKN28, MKN45, and TMK1 cells with or without treatment with the methyltransferase inhibitor 5-aza-dCyd (1 or 5 μmol/L) for 4 days, followed by a 24-hour treatment without or with the histone deacetylase inhibitor trichostatin A (TSA) (50 ng/mL). Values are represented as the mean ± SD of three independent experiments. (B) Demethylation was analyzed by COBRA in MKN28 cells before and after exposure to 5 μmol/L of 5-aza-dCyd for 4 days. Arrows and arrowhead indicate digested fragments (methylated DNA: M) and undigested products (unmethylated DNA: U), respectively. PLC, normal peripheral lymphocytes.


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310–6.