Expression of POT1 is Associated with Tumor Stage and Telomere Length in Gastric Carcinoma

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ABSTRACT

Pot1, a telomere end-binding protein in fission yeast and human, is proposed not only to cap telomeres but also to recruit telomerase to the ends of chromosomes. No study has been performed regarding Pot1 expression status in human cancers. Thus, we examined POT1 mRNA expression in 51 gastric cancer (GC) tissues and evaluated telomere length and 3′ telomeric overhang signals in 20 of the 51 GC tissues. Quantitative reverse transcription-PCR analysis showed that POT1 expression levels in the tumor relative to those in nonneoplastic mucosa (T/N ratio) were significantly higher in stage III/IV tumors than in stage I/II tumors (P = 0.005). Down-regulation of POT1 (T/N < 0.5) was observed more frequently in stage I/II GC (52.4%, 11 of 21) than in stage III/IV GC (23.3%, 7 of 30; P = 0.033), whereas up-regulation of POT1 (T/N > 2.0) was observed more frequently in stage III/IV GC (33.3%, 10 of 30) than in stage I/II GC (9.5%, 2 of 21; P = 0.048). POT1 expression levels showed decreased in accordance with telomere shortening (r = 0.713, P = 0.002). In-gel hybridization analysis showed that 3′ telomeric overhang signals decreased in accordance with decreases in POT1 expression levels (r = 0.696, P = 0.002) and telomere shortening (r = 0.570, P = 0.013).

Reduced POT1 expression was observed in GC cell lines with telomeres shortened by treatment with azidothymidine. In addition, inhibition of Pot1 by antisense oligonucleotides led to telomere shortening as well as inhibition of telomerase activity in GC cells. Moreover, inhibition of Pot1 decreased 3′ overhang signals and increased the frequency of anaphase bridge (P = 0.0005). These data suggest that Pot1 may play an important role in regulation of telomere length and that inhibition of Pot1 may induce telomere dysfunction. Moreover, changes in POT1 expression levels may be associated with stomach carcinogenesis and GC progression.

INTRODUCTION

Telomeres are distinctive structures consisting of a repetitive DNA sequence (TTAGGG) and associated proteins that cap the ends of linear chromosomes. Telomeres enable cells to distinguish chromosomal ends from double-strand breaks in the genome. Mammalian telomeric DNA is mostly composed of double-stranded 5′-TTAGGG-3′ repeats and terminates with a single-stranded overhang of the G-rich strand (1–3). In human somatic cells, telomeres have 500-3000 TTAGGG repeats, but telomeres shorten gradually with age (4–6). In contrast, telomeres of germ line and cancer cells do not shorten, consistent with the behavior of immortal and unicellular organisms. The telomeres of immortal cells are maintained by telomerase, which is able to extend 3′ telomeric overhangs, or by recombination (7–10). Telomerase activity confers cell immortality through stabilization of the chromosome, and it participates in the development of the majority of human cancers. We have shown that telomerase activity occurs in early-stage gastric cancer (GC; Ref. 11) and that telomerase reverse transcriptase expression is required for telomerase activity in the initiation of carcinogenesis in the stomach (12).

A single-stranded telomeric DNA binding protein, protection of telomeres (Pot1), has been identified in fission yeast and human (13). In fission yeast, most cells lacking Pot1 die because of sequence loss and end-to-end chromosomal fusion, although a few survivors emerge that have circularized all three chromosomes, thereby bypassing the requirement for chromosomal end maintenance. Purified fission yeast and human Pot1 proteins bind specifically to the G-rich strand of their own telomeric DNA but not to the complementary C-rich strand or double-stranded telomeric DNA, consistent with a role in binding to the 3′ telomeric overhang at the ends of telomeres in vivo. In Saccharomyces cerevisiae, the single-stranded telomeric DNA binding protein Cdc13 not only caps telomeres but also recruits telomerase to the ends of chromosomes (14, 15). Therefore, Pot1 is thought to be involved in this dual task (13, 16–18). A recent study indicated that each Pot1 binds to one telomeric repeat and coats the entire single-stranded overhang of the telomere in Schizosaccharomyces pombe (18). However, no study has investigated Pot1 expression status and its association with telomere length in human cancers including GC.

We investigated expression of the POT1 gene and its relation to telomere length and 3′ telomeric overhang in GC tissues. Moreover, we studied the relation between POT1 gene expression levels and telomere length in GC cell lines using azidothymidine (AZT) and POT1 antisense oligonucleotides because AZT causes telomere shortening by inhibiting telomerase activity (19–22). In addition, we investigated alteration of 3′ telomeric overhang signals and the frequency of anaphase bridges in GC cells treated with POT1 antisense oligonucleotides.

MATERIALS AND METHODS

Samples. Fifty-one pairs of GC tissues and corresponding nonneoplastic mucosa were studied. Specimens were removed surgically, frozen immediately in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the carcinoma specimens consisted mainly of carcinoma tissue and that the nonneoplastic mucosa showed no invasion by carcinoma cells or significant inflammatory involvement. Histological classification and tumor staging were done according to the Lauren classification system (23) and tumor-node-metastasis (24) classification systems. From among a total 51 GC cases, we randomly selected 20 cases in which high molecular weight DNA was available to evaluate telomere lengths and 3′ telomeric overhang signals.

GC Cell Lines. Two cell lines derived from human GC were used: MKN-28 and MKN-74 derived from well-differentiated adenocarcinomas and kindly provided by Dr. Toshimitsu Suzuki. Both cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MA) in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Quantitative Reverse Transcription (RT)-PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (1 µg) was converted to cDNA with the First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCRs were performed with the SYBR
levels in corresponding nonneoplastic mucosa. mRNA expression levels in GC tissue relative to man's rank correlation test). T/N ratio mined in tumor tissues (T) and corresponding non-neoplastic mucosae (N). Horizontal minun 0.713, T/N ratio stage II/III: 3.84, and for control, complementary (sense) oligonucleotides were synthesized as 5′−CAATGTCTTGAGAACAAAGACAT−3′, and for control, complementary (sense) oligonucleotides were synthesized as 5′−CAATGTCTTGAGAACAAAGACAT−3′. We harvested MKN-28 cells maintained at 37°C under 5% CO2 in RPMI 1640 and 10% fetal bovine serum. Cells were pretreated with 2.5 μM antisense or sense oligonucleotides in Lipofectamine (Invitrogen-Life Technologies, Inc., Carlsbad, CA) for 4 and 8 days by medium exchange and addition of antisense or sense oligonucleotides every 2 days before being used for experiments.

Anaphase Bridges. H&E-stained cultures or tissue sections were examined for anaphase bridges under a light microscope at ×100 magnification. The anaphase bridge index (ABI) was determined by dividing the number of anaphases with bridges by the total number of anaphases. Anaphase bridging was defined as reported previously (26). Two investigators independently scored a minimum of 10 anaphases/sample.

Statistical Analysis. Statistical significance was assessed by Fisher’s exact test, Mann-Whitney U test, Spearman’s rank correlation test, or unpaired t test. Statview 5.0 Macintosh software was used. All tests were two-sided. A P of <0.05 was regarded as statistically significant.

RESULTS

POT1 Expression Levels Increase with Tumor Stage. Expression levels of POT1 were measured by quantitative RT-PCR in the 51 cases of GC. We calculated the ratio of POT1 mRNA expression levels in GC tissues relative to levels in nonneoplastic mucosa (T/N ratio). The T/N ratios were significantly higher in stage III/IV cancers than in stage I/II cancers (P = 0.005, Mann-Whitney U test; Fig. 1A). We considered a T/N > 2.0 to represent up-regulation and a T/N < 0.5 to represent down-regulation. Up-regulation of POT1 was found in 12 (23.5%) of the 51 cases, and down-regulation was found in 18 (35.3%) of the 51 cases. Down-regulation of POT1 was more frequent in stage I/II tumors (52.4%, 11 of 21) than in stage III/IV tumors (23.3%, 7 of 30; P = 0.033, Fisher’s exact test; Table 1),
whereas up-regulation of \( \text{POT1} \) was more frequent in stage III/IV tumors (33.3%, 10 of 30) than in stage I/II tumors (9.5%, 2 of 21; \( P = 0.048 \), Fisher’s exact test; Table 1). In addition, down-regulation of \( \text{POT1} \) was observed preferentially in low T grade (depth of invasion) cancers (\( P = 0.038 \), Fisher’s exact test; Table 1). No association was found between \( \text{POT1} \) expression level and N grade (degree of lymph node metastasis) or histological type (Table 1).

Table 1 Clinical pathological features of gastric cancers (\( n = 51 \)) in relation to \( \text{POT1} \) expression levels

<table>
<thead>
<tr>
<th>Histology</th>
<th>No change (T/N &lt; 0.5)</th>
<th>Down-regulation (T/N = 0.5 – 2.0)</th>
<th>Up-regulation (T/N &gt; 2.0)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal</td>
<td>9 (36.0%)</td>
<td>9 (36.0%)</td>
<td>7 (28.0%)</td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>9 (34.6%)</td>
<td>12 (46.2%)</td>
<td>5 (19.2%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>( T_1,2 )</td>
<td>12 (50.0%)</td>
<td>9 (37.5%)</td>
<td>3 (12.5%)</td>
<td></td>
</tr>
<tr>
<td>( T_3,4 )</td>
<td>6 (22.2%)</td>
<td>12 (44.5%)</td>
<td>9 (33.3%)</td>
<td>0.038</td>
</tr>
<tr>
<td>N-grade(^d)</td>
<td>6 (46.2%)</td>
<td>5 (38.4%)</td>
<td>2 (15.4%)</td>
<td></td>
</tr>
<tr>
<td>Stage(^e)</td>
<td>12 (31.6%)</td>
<td>16 (42.1%)</td>
<td>10 (26.3%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>I, II</td>
<td>11 (52.4%)</td>
<td>8 (38.1%)</td>
<td>2 (9.50%)</td>
<td></td>
</tr>
<tr>
<td>III, IV</td>
<td>7 (23.3%)</td>
<td>13 (43.4%)</td>
<td>10 (33.3%)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

\(^{a}\) T/N ratio = \( \text{POT1} \) mRNA expression levels in GC tissue relative to levels in corresponding nonneoplastic mucosa.

\(^{b}\) According to the Lauren criteria (23).

\(^{c}\) No association.

\(^{d}\) According to the criteria of the tumor-node-metastasis stage classification system (24).

\(^{e}\) By Fisher’s exact test, for down-regulation versus no change and up-regulation.

\(^{f}\) By Fisher’s exact test, for down-regulation and no change versus up-regulation.

Positive Correlation between \( \text{POT1} \) Expression Levels and Telomere Length. For association between \( \text{POT1} \) expression levels and telomere length in GC tissues, telomere length was examined by Southern blotting analysis in 20 of the 51 cases (Fig. 1B). \( \text{POT1} \) expression levels in GC tissue decreased in accordance with telomere shortening (\( r = 0.713, P = 0.002 \), Spearman’s rank correlation test; Fig. 1C).

Positive Correlation between \( \text{POT1} \) Expression Levels and 3’ Telomeric Overhang Signals. 3’ overhang signals were examined by in-gel hybridization analysis in the same 20 GC tissues in which telomere lengths were measured (Fig. 1A). 3’ overhang signals reduced in accordance with reduced \( \text{POT1} \) expression levels (\( r = 0.696, P = 0.002 \), Spearman’s rank correlation test; Fig. 1B) and telomere shortening (\( r = 0.570, P = 0.013 \), Spearman’s rank correlation test; Fig. 1C).

AZT Inhibition of \( \text{POT1} \) Expression in GC Cells. To confirm the positive correlation between \( \text{POT1} \) expression levels and telomere length, we measured \( \text{POT1} \) expression levels in GC cells in which telomeres were shortened by AZT. AZT inhibited telomerase activity and shortened telomeres (Fig. 3, A and B). Telomerase activity was inhibited with AZT by 68% after 5 days and 82% after 10 days in MKN-28 cells and by 23% after 5 days and 94% after 10 days in MKN-74 cells. AZT reduced telomere length from 3.9 to 3.7 Kb after 5 days and to 3.4 Kb after 10 days in MKN-28 cells and from 3.6 to 3.1 Kb after 5 days and to 2.7 Kb after 10 days in MKN-74 cells. Quantitative RT-PCR analysis showed \( \text{POT1} \) expression to be down-regulated in AZT-treated cells (Fig. 3C). Expression of \( \text{POT1} \) in AZT-treated MKN-28 cells was 29% of that in nontreated cells after

Fig. 1. 3’ Telomeric overhang signals were associated with \( \text{POT1} \) expression levels and telomere lengths. A, representative in-gel hybridization assay. The top left panel shows native gel probed with [CCCTAA] 4 , and the top right panel shows denatured gel probed with [CCCTAA] 4 . The bottom left panel shows native gel probed with [TTAGGG] 4 , and the bottom right panel shows denatured gel probed with [TTAGGG] 4 . Signals were expressed relative to the signal in MKN-74 gastric cancer cells. B, \( \text{POT1} \) expression levels in tumor tissues correlate positively with 3’ telomeric overhang signals in gastric cancer tissues (\( r = 0.696, P = 0.002 \) by Spearman’s rank correlation test). C, telomere lengths correlate positively with 3’ telomeric overhang signals in gastric cancer tissues (\( r = 0.570, P = 0.013 \) by Spearman’s rank correlation test).
5 days and 26% after 10 days. And expression of POT1 in AZT-treated MKN-74 cells was 36% of that in nontreated cells after 5 days and 1% after 10 days.

**Inhibition of POT1 Induces Telomere Shortening.** For additional analysis, we examined MKN-28 cells treated with POT1 antisense oligonucleotides. Decreased POT1 expression was confirmed by quantitative RT-PCR in MKN-28 cells after treatment with antisense oligonucleotides (Fig. 4A). Expression of POT1 in antisense oligonucleotide-treated MKN-28 cells was 40% of nontreated cells after 4 days and 34% after 8 days. Treatment with POT1 antisense oligonucleotides resulted in telomerase inhibition (by 58% after 4 days and 83% after 8 days; Fig. 4B) and telomere shortening (from 3.8 to 3.3 Kb after 4 days and to 3.0 Kb after 8 days; Fig. 4C). Treatment with sense oligonucleotides did not produce any alterations in POT1 expression levels, telomerase activity, or telomere length.

**Inhibition of POT1 Reduces 3’ Telomeric Overhang Signals.** To test whether POT1 expression levels are associated with 3’ overhang signals, 3’ telomeric overhang signals were examined by in-gel hybridization in MKN-28 cells treated with POT1 antisense oligonucleotides. A reduction in 3’ overhang signals was found in antisense

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**Fig. 3. POT1 expression levels in relation to telomere shortening.** A, reduced telomerase activity in MKN-28 and MKN-74 cells is observed after 5 and 10 days’ treatment with azidothymidine (AZT). IC = internal control (36 bp). B, Telomere shortening is observed in MKN-28 and MKN-74 cells after 5 and 10 days’ treatment with AZT. C, quantitative reverse-transcription-PCR analysis showed reduced POT1 expression in MKN-28 and MKN-74 cells treated with AZT. POT1 expression levels are the mean ± SD and relative to levels in nontreated cells.

**Fig. 4. Telomere shortening, telomerase inhibition and the reduction of 3’ telomeric overhang signals in association with reduced expression of POT1 in antisense oligonucleotides-treated MKN-28 cells.** A, reduction of POT1 expression was confirmed in MKN-28 cells after antisense oligonucleotide treatment for 4 and 8 days. B, reduced telomerase activity was detected in antisense oligonucleotides-treated MKN-28 cells, whereas telomerase activity was not changed in sense oligonucleotides-treated MKN-28 cells. C, telomere shortening was detected in MKN-28 cells treated with antisense oligonucleotides. D, inhibition of Pot1 reduced 3’ telomeric overhang signals. The top left panel shows native gel probed with [CCCTAA]_4, and the top right panel shows denatured gel probed with [CCCTAA]_4. The bottom left panel shows native gel probed with [TTAGGG]_4, and the bottom right panel shows denatured gel probed with [TTAGGG]_4. Signals were expressed relative to the signal in the nontreated MKN-28 gastric cancer cells and normalized to the amount of DNA (10 μg) loaded based on denatured gels.
We showed expression levels of POT1 from degradation and DNA repair activities (13, 16) telomeric overhang/H11032.

**DISCUSSION**

Pot1 is thought to have two functions. One is mediating recruitment of telomerase, and the other is protecting the 3′ telomeric overhang from degradation and DNA repair activities (13, 16–18). We studied POT1 mRNA expression in GC tissues with respect to both functions. We showed expression levels of POT1 in the GC tissues relative to levels in nonneoplastic mucosae to be significantly associated with tumor stage and that up-regulation of POT1 occurs preferentially in late-stage GC. Pot1 is thought to protect telomeres (13). In fact, inhibition of POT1 by antisense oligonucleotides led to an increase in the frequency of anaphase bridge in MKN-28 cells (51). Thus, inhibition of Pot1 is associated with telomere dysfunction. Previous studies in a mouse model revealed that severe telomere dysfunction impaired tumor progression (26–31). Severe telomere dysfunction is shown to be reduced in advanced tumors that survive after crisis (26, 32, 33). Thus, up-regulated POT1 may participate in protection of the telomere ends in late-stage GC. In addition, we found POT1 mRNA expression levels to be associated with telomere length, as well as 3′ telomeric overhang signals in GC tissues. However, we did not find significant association between telomere length or 3′ telomeric overhang signals and tumor stage (data not shown). High-level POT1 expression, as well as telomerase activity, may be required for maintenance of telomere function.

Telomere dysfunction appears to occur in cancer precursor lesions and increase the frequency of genetically initiated neoplasms (26, 30, 31, 34–39); therefore, in early-stage GC after neoplasm initiation, down-regulation of POT1 may occur preferentially. We showed that down-regulation of POT1 was observed preferentially in low T grade cancers, which might also reflect telomere dysfunction in early-stage GC. Approximately half of our GC patients (21 of 51) showed no changes in the expression level of POT1, perhaps because telomere dysfunction is circumvented by chromosomal rearrangement such as in centric and ring chromosomes in these patients.

The ABI did not correlate with POT1 expression levels, telomere length, or 3′ telomeric overhang signals. Anaphase bridges are chromatin bridges that are not resolved after anaphase, and they result in breakage-fusion-bridge cycles that produce rapid and widespread changes in the gene (40–46). Anaphase bridges are a hallmark of telomere dysfunction (26) but form not only as a result of defects in telomere structure or length (6, 34) but also as a result of defects in DNA replication (47), recombinations (48), or translocations that introduce a second centromere into the chromosome (46, 49). Therefore, we could not show significant association between ABI and POT1 expression levels, telomere length, or 3′ telomeric overhang signals.

It was reported recently that the 3′ telomeric overhang is shortened at senescence and that progressive overhang loss occurred in cells that avoided senescence through inactivation of p53 and Rb (50). Evidence indicates that 3′ telomeric overhang shortening is the result of continuous cell division and that it is associated with telomere shortening. We showed telomere length to be associated with 3′ telomeric overhang signals, consistent with previously reported findings (50).

To confirm the association between POT1 expression levels and telomere length, we measured telomere length in AZT-treated GC cells and showed reduced POT1 expression as well as telomere shortening. Loayza et al. (51) also reported the amount of Pot1 to be correlated with telomere length. Our data support their findings. However, we cannot fully rule out the possibility that the down-regulation of POT1 may have been because of a direct effect of AZT or other factors. We examined association between POT1 expression levels and telomere length, 3′ telomeric overhangs, and the frequency of anaphase bridges using GC cells treated with POT1 antisense oligonucleotides. The inhibition of POT1 by antisense oligonucleotides was found to shorten the telomere, reduce the 3′ overhang signals, and increase the frequency of anaphase bridges. Colgin et al. (52) reported that overexpression of POT1 led to telomere elongation. In yeast, Pot1-like protein Cdc13 recruits telomerase to the 3′ telomeric overhang (14); thus, inhibition of POT1 may lead to telomere shortening through inhibition of recruitment of telomerase to the 3′ telomeric overhang. Furthermore, our study showed that inhibition of telomerase activity occurred via POT1 antisense oligonucleotides. We are unable to explain this phenomenon at the present. However, inhibition of telomerase activity by POT1 antisense oligonucleotides may participate partly in telomere shortening. We examined the viability of antisense-treated MKN-28 cells (4- and 8-day treatments) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and observed no change in the viability of treated cells compared with nontreated cells (data not shown). Thus, it seems there is no association between telomerase inhibition and decreased viability of cells. We found inhibition of POT1 by antisense oligonucleotides to reduce 3′ overhang signals. Therefore, it is possible that POT1 expression levels may generally depend on the size of the 3′ telomeric overhang. However, it remains a possibility that the reduction of 3′ overhang signals may be because of end-to-end fusion. Indeed, inhibition of POT1 led to an increase in the frequency of anaphase bridges. In this study, no signal was present at the position of the larger terminal fragments representing the fused telomeres (53). Therefore, at least some of the loss of 3′ overhangs in GC cells must have taken place on unfused chromosome ends, and end-to-end fusion may have little effect on 3′ overhang signals.

Loayza et al. (51) observed telomere elongation using Pot1 mutant lacking the oligosaccharide/oligonucleotide-binding (OB) fold required for DNA binding. We are unable to fully explain the discrep-
ancy between the Loayza et al. (51) findings and those of Colgin et al. (52) along with ours. There are several possible explanations: we and Colgin et al. (52) altered whole expression levels of wild-type POT1 with antisense oligonucleotides and expression vectors (52); thus, the discrepancy may be because of methodological differences. We did not perform any mutation analysis in the oligosaccharide/oligonucleotide-binding (OB) fold of Pot1 in the present study. This should be done in further study. Although telomere length may be regulated by the interaction between Pot1 and TRF1 complex, we did not examine TRF1 expression status in the present study. In yeast, the interaction between Cdc13 and telomerase has been previously described (14), but in human cells, the interaction between Pot1 and telomerase has not been examined. We presume that such investigation is most essential to understanding the function of Pot1.

Adequate telomere length, telomerase activity, and T-loop formation are required for maintenance of telomere function, and when only one mechanistic factor is compromised such as in a lack of functional telomerase or telomere shortening, the other components of the capping system can compensate (16). The association we observed between telomere length and telomerase activity indicates that Pot1 may play an important role in the maintenance of telomere function. Telomere dysfunction leads to genetic instability at the chromosome ends, and such instability is associated with the initiation of carcinogenesis (26, 30, 31, 34). One mechanistic factor is compromised such as in a lack of functional interaction between Pot1 and TRF1 complex, we did not examine POT1 expression to reflect telomere dysfunction and that they may serve as a useful screening tool for identifying individuals at greatest risk of carcinogenesis. Additional studies are needed to establish Pot1 as a clinical indicator of cancer risk.

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