Basic Research Paper

Changes of alternative splicing variants of human telomerase reverse transcriptase during gastric carcinogenesis

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Key words: gastric neoplasm, precancerous lesion, telomerase reverse transcriptase, alternative splicing, polymerase chain reaction, diagnosis

Background and Objective: The expression of human telomerase reverse transcriptase (hTERT) is positively correlated to the activity of telomerase. Alternative splicing exists in the transcription of hTERT and special splicing patterns may change during tumor progression. This study intended to reveal the changes of hTERT alternative splicing pattern in gastric carcinogenesis.

Methods: Three alternative splicing sites (α, β, γ) were selected to design primers. The expression of eight hTERT alternative splicing variants (ASVs) in 18 specimens of normal gastric mucosa, 20 specimens of precancerous lesions and 19 specimens of gastric cancer was detected by semi-nested reverse transcription-polymerase chain reaction (RT-PCR). The expression of β site-remaining ASV (β+ hTERT mRNA) in precancerous lesions and gastric cancer tissues was detected by SYBR Green real-time RT-PCR.

Results: The expression of β+ hTERT mRNA was significantly higher in gastric cancer than in precancerous lesions and normal mucosa (94.7% vs. 40.0% and 0, p < 0.05). The positive rates of other ASVs were not different among the three groups. The positive rates of β-deletion ASV were 72.2% in normal mucosa, 95.0% in precancerous lesions and 100.0% in gastric cancer. The positive rates of β+ hTERT mRNA (including αβγ hTERT mRNA, α-deletion ASV, γ-deletion ASV) were 11.1% in normal mucosa, 40.0% in precancerous lesions and 94.7% in gastric cancer (p < 0.05).

The mRNA level of β+ hTERT was 6.99 times higher in gastric cancer than in precancerous lesions. Conclusions: hTERT alternate splicing pattern changes during gastric carcinogenesis.

β+ hTERT mRNA is expressed increasingly during gastric carcinogenesis and may provide useful information for the diagnosis of gastric cancer or precancerous lesions.

Gastric cancer is one of the most common malignancies in the world. In the past few years, studies have found that the expression of certain molecules, such as hTERT, RAR-β, p53, CD44, Cyclin E, APC, TGFβ and c-erB2, was altered in multiple stages of the development of gastric carcinogenesis.1-5 Human telomerase RNA is expressed in a wide ranges of tissues, including both normal and abnormal ones. The activity of telomerase is closely related to the development of gastric cancer and proliferation of cancer cells. Therefore, detecting telomerase activity could be a useful tool to increase diagnostic sensitivity for gastric cancer and precancerous lesions.6 hTERT is the rate limiting factor of telomerase that determines the activity of telomerase. The expression of hTERT is positively correlated to the activity of telomerase, and thus can be a surrogate parameter for the activity of telomerase.7-10 In situ hybridization detection revealed that positive rate of hTERT mRNA in gastric cancer was significantly higher than those in adjacent tissue and benign gastric mucosal lesion.11 Therefore, detecting the expression of hTERT mRNA or hTERT protein can be an alternative method to measure telomerase activity. hTERT undergoes alternative splicing in the process of transcription, which has an important influence on the telomerase activity.12,13 In order to provide information for the diagnosis of gastric cancer and precancerous lesion, we measured the expression of hTERT ASVs in normal gastric mucosa, gastric precancerous lesion and gastric cancer by designing site-specific primers at three splicing sites (α, β and γ) of hTERT pre-mRNA.

Materials and Methods

Materials. 18 specimens of normal gastric mucosa, 20 specimens of precancerous lesions (chronic atrophic gastritis with moderate to severe dysplasia or moderate to severe intestinal metaplasia) and 19 specimens of gastric cancer tissues were obtained from the department of gastroscopy of Tianjin Medical University General Hospital (March to June in 2006) and confirmed by department of pathology of Tianjin Medical University. Specimens were put into...
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Methods. Total RNA extraction and RT-PCR. Total RNAs were extracted from all the specimens using Trizol™ (Invitrogen), and the RNAs integrity was tested by 1% agarose gel electrophoresis. Eligible RNAs were then stored at -80°C. A RT-PCR test was run under the following conditions: 5 min at 70°C and then 60 min at 37°C and finally 15 min at 70°C. cDNAs were stored at -20°C.

Semi-nested RT-PCR for the detection of hTERT ASVs. Sequence of primers used in the amplification of hTERT ASVs were shown in Table 1. Primers sites were shown in Figure 1. Sequences of primers were designed in accord with alternative splicing sites of hTERT (AF0181670),14 and were designed and analyzed with Oligo 6.0. Each hTERT ASV was amplified in two PCRs. Reactions were conducted under the following conditions: 2 min at 95°C for initial denaturation; and 30 cycles of 30 sec at 95°C, 30 sec at 60°C and 40 sec at 72°C; and finally 10 min at 72°C.

Purification, cloning and sequencing of PCR products. Positive bands of PCR products were cut and then extracted using an extraction kit. Extracted products were then connected to pGMM-T Easy Vector (by Promega) for the amplification of positive clones. Vectors were then extracted. Target fragments were cleaved by restriction endonuclease EcoR I (by Takara Biotechnology [Dalian] Co., Ltd.). Products were then verified by agarose gel electrophoresis. Verified products were submitted to Shanghai Sangon Biological Engineering Co., Ltd., for sequencing.

Fluorescent real-time RT-PCR for quantitative measurement of β⁺ hTERT mRNA. β⁺ ASV (that is, β site-remaining ASVs) was amplified with PCR. Sequences and sites of primers were designed and analyzed with DNASTar and Oligo6.0 software (Table 2 and Fig. 2). PCR products were all β site-remaining ASVs, which helped exclude the possibility that β site-deleted ASVs might exist in these PCR products. Six specimens of β⁺ ASV-positive precancerous lesion and six specimens of β⁺ ASV-positive gastric cancer tissues were selected for quantitative measurement of β⁺ ASVs via real-time RT-PCR.

Statistical analysis. SPSS 13.0 statistical software was used in the analysis. Data were analyzed with chi-square test. Differences were considered statistically significant when p < 0.05.

Results

Semi-nested RT-PCR for the detection of hTERT ASVs. Expression of αβ⁺γ⁺ hTERT mRNA was not detected in normal gastric mucosa, while a positive rate of αβ⁺γ⁺ hTERT mRNA in gastric cancer tissue was significantly higher than in precancerous lesion (p < 0.05). Positive rates of β site-deletion ASV in normal gastric mucosa, precancerous lesion and gastric cancer tissue

| Table 1 Primers for alternative splicing variants (ASVs) of human telomerase reverse transcriptase (hTERT) |
|---|---|---|
| Gene | Sequences | Position |
| ex6-F | 5'-ATG TCA ACC GGT AGC-3' | 2191-2210 |
| ex11-R | 5'-GAA ATG CCA CCA CTG TCC GC-3' | 2756-2778 |
| Adel-F | 5'-CTG CAG GAG ACC CCG C-3' | 2167-2186, 2223-2228 |
| Gdel-R | 5'-GTA GCA CTT GCC CCT GAT GGC-3' | 2702-2710, 2900-2913 |
| ex7-F | 5'-CTG AGC TGT ACT TGT TCA AGG ACA AGC-3' | 2394-2412 |
| ex8.9-R | 5'-AGG TCC GGG CAT AGC TGA GGA CAG-3' | 2511-2531 |
| Bdel-F | 5'-GCC TTC AAG AGC CAC GTC CTA CGG-3' | 2327-2342, 2525-2531 |
| Bdel-R | 5'-GCA CTG GAC GTA GGA CGT GGC T-3' | 2336-2342, 2525-2540 |
Table 2  Primers for β-site remaining (β⁺) hTERT mRNA and GADPH (NM_002046)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
<th>Position</th>
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<tr>
<td>β⁺-F</td>
<td>5’-AGG TCA TCG CCA GCA TCA AAG-3’</td>
<td>2256-2259</td>
</tr>
<tr>
<td>β⁺-R</td>
<td>5’-AGG GCT GGA GCT CTG TCA AGG TAG-3’</td>
<td>2347-2370</td>
</tr>
<tr>
<td>GADPH-F</td>
<td>5’-GCT GAA GCT CCG AGT CAA CCG A-3’</td>
<td>111-132</td>
</tr>
<tr>
<td>GADPH-R</td>
<td>5’-GAG GGA TCT CCG TGC TGG AAG A-3’</td>
<td>329-350</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>α-deletion site</th>
<th>β-deletion site</th>
<th>γ-deletion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>12</td>
<td>β⁺-F β⁺-R</td>
</tr>
</tbody>
</table>

Figure 2. The position of primers for β⁺ ASV.

were 72.2%, 95.0% and 100.0%, respectively. The expression of αβγ-deletion ASV was not detected in our study. As for the other five ASVs, there were no statistically significant differences of positive rates of these ASVs in normal gastric mucosa, precancerous lesion and gastric cancer tissue (p > 0.05). Positive rates of β site-deletion ASVs (β-deletion ASV, αβ-deletion ASV and βγ-deletion ASV) in normal gastric mucosa, precancerous lesion and gastric cancer tissue were 83.3%, 95.0% and 100.0%, respectively. No statistically significant differences were found in the positive rates between these groups (p > 0.05). In gastric carcinogenesis, the positive rate of β⁺ ASV (αβ⁺γ⁺ hTERT mRNA, α-deletion ASV, β-deletion ASV and αγ-deletion ASV) increased. The positive rate of β⁺ ASV was 11.1% in normal gastric mucosa, 40.0% in precancerous lesion and 94.7% in gastric cancer tissue. The differences in the three groups were statistically significant (p < 0.05). The results of hTERT ASVs sequencing showed complete consistence with targeted sequences.

Quantitative measurement of β⁺ hTERT mRNA with fluorescent real-time RT-PCR. RT-PCR test. β⁺ hTERT mRNA was absent in normal gastric mucosa, while it was found in 6 out of 20 specimens of precancerous lesions and 16 out of 19 specimens of gastric cancer tissues. The differences these groups were statistically significant (p < 0.05).

Quantitative measurement with fluorescent real-time RT-PCR. Amplification curves and standard curves of GADPH and hTERT mRNA were shown in Figure 3. β⁺ hTERT mRNA positive specimens of precancerous lesions and gastric cancer tissues were selected for quantitative detection with fluorescent real-time RT-PCR. The results were shown in Figures 4 and 5. Data were conducted by 2^-ΔΔCT method. The result showed that expression of β⁺ hTERT mRNA in gastric cancer was 6.99 times higher than in precancerous lesions.

Discussion

hTERT is the rate-limiting subunit of telomerase, the activity of which is regulated by hTERT at transcriptional level. Alternative splicing exists in the process of hTERT transcription. Alternative splicing causes numerous protein variants with different biological properties, including protein binding capacity, intracellular localization, activity of enzyme, stability of protein and post-translational modification. Studies have shown that alternative splicing may inactivate protein, modify its functions or produce protein with novel functions. Alternative splicing and subsequent functional modification in protein variants are the critical mechanism that generates functional diversity of proteome.16-19 Fujiwara et al.20 found that the expression of hTERT mRNA were low in TKB-4 and TK-20 cell lines, and the splicing patterns of hTERT mRNA in these cells were different. In TKB-4 cell lines, the α⁺β⁺ ASV (both α and β sites remain) accounts for 15.4% of all hTERT mRNA. While α⁺β⁺ ASV was not detected in TK-20, and β⁺ ASV was noticed expressed. These results indicated that alternative splicing of hTERT mRNA had an effect on the activity of telomerase. The expression of hTERT is a key factor in carcinogenesis. Studies on patients with gastric cancer and chronic gastritis found that hTERT was expressed in most precancerous lesions, which suggested that the expression of hTERT occurred in the early stage of gastric carcinogenesis and it might play a role in gastric carcinogenesis and its subsequent progression.21

The hTERT gene consists of 16 exons and 15 introns, while the hTERT protein contains one telomerase-specific motif and seven reverse transcriptase motifs. These motifs are prerequisites of hTERT protein. There are at least 13 alternative splicing sites in hTERT mRNA, of which three are deletion sites (α, β and γ) and ten are insertion sites. To investigate the changes in alternative splicing patterns of hTERT in gastric carcinogenesis, we selected three deletion sites for our study.

The products of α-deletion and γ-deletion, ASVs only lost a small portion of hTERT protein. Thus experts speculated that these ASVs might have negative effect on telomerase activity.10,23 Yi et al.24 have found that α-deletion ASV was overexpressed in immortalized cells and some tumor cell lines (SW39, H1299 and DU145), which can inhibit telomerase activity, while β-deletion and αβ-deletion ASVs showed no effect on the telomerase activity. Kotoula et al.25 found that low-grade astrocytoma tissues mainly expressed α-deletion and β-deletion ASVs, while in high-grade astrocytoma tissues, especially grade IV astrocytoma, always expressed α⁺β⁺ ASV; however α-deletion ASV was not expressed. These findings suggested that expression of α-deletion ASV decreased and activity of telomerase increased with tumor progression. The expression of α-deletion ASV in astrocytoma might inhibit the activity of telomerase. Hence, α-deletion ASV, whose expression might be suppressed in carcinogenesis, has a downregulating effect on the activity of telomerase. In our study, we found α-deletion and γ-deletion ASVs had no statistic significance in the three groups of specimens, which is consistent with Barclay's26 findings. However, we were not able to prove that they are inhibitors of the activity of telomerase. This may because α-deletion and γ-deletion ASVs could have a dose-dependent downregulating effect on the activity of telomerase. However, our study didn't provide information regarding their expression levels. Barclay26 found even normal tissues adjacent to gastric cancers were expressed hTERT mRNA; while no expression of hTERT mRNA was detected in normal gastric mucosa in our study. For one thing, the
Figure 3. The amplification curve and standard curve of β+ ASV.
normal gastric mucosa specimens used in our study were obtained from normal people, rather than cancer-adjacent tissue. Different specimens may have led to different results. Moreover, the specimens used in our study were obtained by gastroscopy, while in the references specimens were obtained during gastric cancer surgeries. The quantity of specimen might also influence the results. Last but not the least, the LightCycler TeloTAGGhTERT Quantification Kit was used in their studies, while we conducted RT-PCR, which might also explain the difference in results.

We found β-deletion ASV was expressed in most of the specimens, including normal gastric mucosa, precancerous lesion and gastric cancer tissue, which was consistent with findings of a related reference. As for αβ-deletion ASV, βγ-deletion ASV and αγ-deletion ASV, positive rates of these ASVs were low in normal
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Figure 5. The amplification curve and melting curve of β1* ASV.
Expression of hTERT ASVs in gastric carcinoma, precancerous lesions and normal gastric mucosa detected by semi-nested RT-PCR

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>αβγ-ASV (cases %)</th>
<th>αβ-ASV (cases %)</th>
<th>β-ASV (cases %)</th>
<th>γ-ASV (cases %)</th>
<th>αβ-γ-ASV (cases %)</th>
<th>αβ-γ-ASV (cases %)</th>
<th>β-γ-ASV (cases %)</th>
<th>αγ-ASV (cases %)</th>
<th>αβ-γ-ASV (cases %)</th>
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</thead>
<tbody>
<tr>
<td>Normal gastric mucosa</td>
<td>18</td>
<td>4 (22.2)</td>
<td>17 (94.4)</td>
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<tr>
<td>Precancerous lesions</td>
<td>20</td>
<td>7 (35.0)</td>
<td>14 (70.0)</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Gastric cancer</td>
<td>19</td>
<td>12 (63.2)</td>
<td>14 (73.7)</td>
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*p < 0.05, vs. normal gastric mucosa; **p < 0.05, vs. precancerous lesions.

Changes of alternative splicing variants of human telomerase reverse transcriptase during gastric carcinogenesis

Table 3

References