Changes of the Alternative Splicing Variants of Human Telomerase Reverse Transcriptase during Gastric Carcinogenesis

Jin-Heng Xu^a Yu-Chuan Wang^b Xin Geng^a Yan-Yun Li^a Wei-Ming Zhang^a

^aDepartment of Biochemistry and Molecular Biology, Tianjin Medical University, and ^bTianjin Institute of Ophthalmology, Tianjin, China

Key Words

Alternative splicing • Precancerous lesion • Gastric cancer • Telomerase reverse transcriptase

Abstract

Objective: We attempted to reveal the changes of the human telomerase reverse transcriptase (hTERT) alternative splicing pattern in gastric carcinogenesis. **Methods:** Three alternative splicing sites (α , β , γ) were selected and designed PCR primer. The expression of 8 hTERT alternative splicing variants (ASVs) in normal gastric mucosa, precancerous lesions and gastric cancer were detected by seminested RT-PCR. The expression of β -site remaining ASV (β^+ ASV) in specimens of precancerous lesions and specimens of gastric cancer was detected by SYBR Green real-time PCR. **Results:** The positive rate of $\alpha^+\beta^+\gamma^+$ ASV 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 3 groups ($p > 0.05$). The positive rates of β^+ ASVs (including $\alpha^+\beta^+\gamma^+$ ASV, α -deletion ASV, γ -deletion ASV, $\alpha\gamma$ -deletion ASV) were 11.1% in normal mucosa, 40.0% in precancerous lesions and 94.7% in gastric cancer ($p < 0.05$). SYBR Green real-time RT-PCR showed that the expression level of β^+ ASV was 6.99 times higher in gastric cancer than in precancerous lesions. **Conclusion:** hTERT alternative splicing pattern is different dur-

ing gastric carcinogenesis. β^+ ASV was widely expressed in gastric carcinogenesis and may provide some information for diagnosis of gastric cancer or precancerous lesions.

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Introduction

Gastric cancer is one of the most common malignancies worldwide. Previous studies indicated that there were some changes of gene expression in gastric cancer progression, such as human telomerase reverse transcriptase (hTERT), RAR- β , p53, CD44, cyclin E, APC, TGF- β , c-erbB2, etc. [1–5]. Telomerase may play a critical role in the rate-limiting step in cancer. Telomerase activity is associated with cancer cell proliferation of gastric carcinogenesis. Therefore, the detection of telomerase activity may be useful for early diagnosis of cancer [6]. Human telomerase RNA is expressed extensively in normal tissues and abnormal tissues. hTERT is a catalytic subunit of the holoenzyme complex and is a rate-limiting factor of telomerase activity. The expression of hTERT is positively correlated with telomerase activity and could represent the telomerase activity [7–10]. Therefore, detecting the expression of hTERT mRNA or protein may be an alternative method of telomerase activity. Alternative splicing exists in the transcription of hTERT

Table 1. Patients' characteristics

No.	Sex	Age	Diagnosis	No.	Sex	Age	Diagnosis
1	F	29	normal	29	F	66	chronic atrophic gastritis with severe intestinal metaplasia
2	M	34	normal	30	M	72	chronic atrophic gastritis with moderate intestinal metaplasia
3	F	36	normal	31	M	35	chronic atrophic gastritis with moderate intestinal metaplasia
4	F	28	normal	32	F	78	chronic atrophic gastritis with severe intestinal metaplasia
5	F	24	normal	33	M	65	chronic atrophic gastritis with moderate intestinal metaplasia
6	M	29	normal	34	M	54	chronic atrophic gastritis with severe intestinal metaplasia
7	M	38	normal	35	F	49	chronic atrophic gastritis with severe atypical hyperplasia
8	F	33	normal	36	F	33	chronic atrophic gastritis with severe atypical hyperplasia
9	M	35	normal	37	M	42	chronic atrophic gastritis with moderate atypical hyperplasia
10	F	27	normal	38	F	76	chronic atrophic gastritis with moderate atypical hyperplasia
11	F	33	normal	39	F	59	adenocarcinoma
12	M	38	normal	40	F	78	adenocarcinoma
13	F	33	normal	41	F	81	adenocarcinoma
14	F	26	normal	42	M	55	adenocarcinoma
15	M	39	normal	43	M	62	adenocarcinoma
16	M	40	normal	44	F	79	adenocarcinoma
17	F	34	normal	45	M	72	adenocarcinoma
18	F	37	normal	46	F	63	adenocarcinoma
19	M	45	chronic atrophic gastritis with moderate intestinal metaplasia	47	M	59	adenocarcinoma
20	M	39	chronic atrophic gastritis with severe intestinal metaplasia	48	M	60	adenocarcinoma
21	F	44	chronic atrophic gastritis with moderate intestinal metaplasia	49	F	67	adenocarcinoma
22	F	29	chronic atrophic gastritis with severe intestinal metaplasia	50	M	68	adenocarcinoma
23	M	33	chronic atrophic gastritis with moderate intestinal metaplasia	51	F	79	adenocarcinoma
24	M	56	chronic atrophic gastritis with moderate intestinal metaplasia	52	M	65	adenocarcinoma
25	F	60	chronic atrophic gastritis with severe intestinal metaplasia	53	F	58	adenocarcinoma
26	M	69	chronic atrophic gastritis with moderate intestinal metaplasia	54	M	69	adenocarcinoma
27	M	45	chronic atrophic gastritis with severe intestinal metaplasia	55	F	60	adenocarcinoma
28	F	67	chronic atrophic gastritis with moderate intestinal metaplasia	56	F	55	adenocarcinoma
				57	M	79	adenocarcinoma

and is associated with telomerase activity [11–13]. Three alternatively spliced sites of hTERT pre-mRNA were selected in this study. The primers were designed according to the three spliced sites α , β , γ . We detected the expression of hTERT alternative splicing variants (ASVs) in normal gastric mucosa, precancerous lesions and gastric cancer and we hope that it may provide some information for the diagnosis of precancerous lesions or gastric cancer.

Materials and Methods

Tissue Samples

All the specimens were obtained from Tianjin Medical University General Hospital (from March to June 2006). The samples included 18 normal gastric mucosa specimens, 20 precancerous lesions of gastric specimens and 19 gastric cancer specimens (detailed

information is shown in table 1). After obtaining the specimens, they were quickly put into liquid nitrogen and stored at -80°C .

Total RNA Isolation and RT-PCR

Frozen specimens were quickly put into a freezing microtone (-30°C) and were sectioned while frozen. All the frozen sections were put on slides and stained with toluidine blue. Of every two sections, one section was selected and stained with HE in order to find the position of target cells. Sections stained with toluidine blue were put on an inverted microscope and target cells found according to HE sections. Then needles of a sterile syringe (1 ml) were used to remove connective tissue and other nontarget cells. Sections with target cells were quickly stored at -80°C .

Total RNAs were extracted from the frozen sections with target cells using TRIzol™ (Invitrogen Life Technologies). The final RNA preparations were quantified by absorbance analysis at 260 nm and tested by 1% agarose gel electrophoresis. 2 μg of total RNA was reverse transcribed in a 25- μl reaction volume using oligodT (Promega) and M-MLV (Promega) reverse transcriptase and the reaction buffer provided in the Reverse Transcription kit (Promega).

Fig. 1. Three alternative splicing sites lead to 8 ASVs. The α -site deletion causes a 36-base deletion and the β -site deletion results in a 182-base deletion in exon 7 and 8. The γ -site deletion induces a 189-base deletion in exon 11. The Adel-F, ex6-F, ex7-F and Bdel-F primers were forward primers, and ex11-R, ex8.9-R, Bdel-R and Gdel-R were reverse primers.

Table 2. Primers for hTERT mRNA

Name	Primer	Position
ex6-F	5'-ATG TGA CGG GCG CGT ACG AC-3'	2191-2210
ex11-R	5'-GGA AGT TCA CCA CTG TCT TCC GC-3'	2756-2778
Adel-F	5'-CTG CAG GAG ACC AGC CCG C-3'	2167-2186, 2223-2228
Gdel-R	5'-GTA GGA CTT GCC CCT GAT GCG-3'	2702-2710, 2900-2913
ex7-F	5'-CTG AGC TGT ACT TTG TCA AGG ACA GG-3'	2394-2412
ex8.9-R	5'-AGG TCC GGG CAT AGC TGA GGA AG-3'	2511-2531
Bdel-F	5'-GCC TTC AAG AGC CAC GTC CTA CG-3'	2327-2342, 2525-2531
Bdel-R	5'-GCA CTG GAC GTA GGA CGT GGC T-3'	2336-2342, 2525-2540

Seminested RT-PCR Testing hTERT ASVs

The primers of hTERT ASVs are shown in table 2 and figure 1. All the primers were designed according to hTERT (AF0181670) alternative splicing sites [14]. The initial step of RT-PCR was 2 min at 95°C for incubation. Cycles (n = 30) consisted

of a 30-second denaturation phase at 95°C, a 30-second annealing at 60°C and a 40-second extension at 72°C. The final phase was at 72°C incubation for 10 min. The positive products of seminested RT-PCR were reclaimed and sequenced.

Fig. 2. The position of primers for GAPDH and β^+ ASV.

Table 3. Primers for GAPDH (NM_2046) and β^+ ASV

Name	Sequence	Position
β^+ -F	5'-AGG TCA TCG CCA GCA TCA TCA AAC-3'	2236–2259
β^+ -R	5'-ACG GCT GGA GGT CTG TCA AGG TAG-3'	2347–2370
GAPDH-F	5'-GGT GAA GGT CGG AGT CAA CGG A-3'	111–132
GAPDH-R	5'-GAG GGA TCT CGC TCC TGG AAG A-3'	329–350

Table 4. Positive rates of hTERT ASVs in gastric carcinoma, precancerous lesions and normal gastric mucosa by seminested RT-PCR

Groups	$\alpha^+\beta^+\gamma^+$ ASV*, %	α -Deletion ASV, %	β -Deletion ASV, %	γ -Deletion ASV, %	$\alpha\beta$ -Deletion ASV, %	$\beta\gamma$ -Deletion ASV, %	$\alpha\gamma$ -Deletion ASV, %	$\alpha\beta\gamma$ -Deletion ASV, %
Normal gastric mucosa	0.0 (0/18)	16.7 (3/18)	72.2 (13/18)	0.0 (0/18)	33.3 (6/18)	11.1 (2/18)	5.6 (1/18)	0.0 (0/18)
Precancerous lesions	40.0 (8/20)	15.0 (3/20)	95.0 (19/20)	5.0 (1/20)	50.0 (10/20)	25.0 (5/20)	25.0 (5/20)	0.0 (0/20)
Gastric cancer	94.7 (18/19)	10.5 (2/19)	100.0 (19/19)	5.3 (1/19)	42.1 (8/19)	15.8 (3/19)	10.5 (2/19)	0.0 (0/19)

Numbers are given in parentheses. * $p < 0.05$.

SYBER Green Real-Time RT-PCR Assay of β^+ ASVs

β^+ ASVs (β -site remaining ASVs) were amplified by RT-PCR. The primers are shown in table 3 and figure 2. RT-PCR products include all β -site-reserved ASVs. Six β^+ ASV precancerous lesion specimens and 6 gastric cancer specimens were selected and identified by SYBER Green real-time RT-PCR. The real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ relative quantization method according to the manufacturer's directions.

Results

Results of Seminested RT-PCR

The results of hTERT ASVs are shown in table 4 and figure 3. $\alpha^+\beta^+\gamma^+$ ASV was absent in normal gastric mucosa, and its positive rate was higher in gastric cancer than that in precancerous lesions ($p < 0.05$). β -Deletion ASVs were expressed in normal gastric mucosa, precancerous lesions and gastric cancer, and the positive rates of β -deletion ASVs, respectively, were 72.2, 95.0 and 100%.

$\alpha\beta\gamma$ -deletion ASVs were not detected in this study and another 5 kinds of ASVs had no statistical significance among normal gastric mucosa, precancerous lesions and gastric cancer ($p > 0.05$). The positive rates of β -site deletion ASV expression (including β -deletion ASV, $\alpha\beta$ -deletion ASV, $\beta\gamma$ -deletion ASV) were 83.3, 95.0 and 100.0%, respectively, in normal gastric mucosa, precancerous lesions and gastric cancer, but the differences had no statistical significance in the above three groups. The positive rates of β^+ ASV expression (including $\alpha^+\beta^+\gamma^+$ ASV, α -deletion ASV, γ -deletion ASV, $\alpha\gamma$ -deletion ASV) were, respectively, 11.1, 40.0 and 94.7%, and the differences in the three groups were statistically significant ($p < 0.05$). The results of all hTERT ASV sequences were identical with the target sequences.

Result of SYBER Green Real-Time RT-PCR of β^+ ASV

β^+ ASV was not detected in normal gastric mucosa by PCR. Of 20 precancerous lesion specimens, 6 expressed

Fig. 3. Representative gel pictures of seminested RT-PCR of hTERT ASVs. N = Normal gastric mucosa; P = precancerous lesions; C = gastric cancer.

β^+ ASV, while of 19 gastric cancer specimens, 16 expressed β^+ ASV. The differences were statistically significant ($p < 0.05$). β^+ ASV in precancerous lesions and gastric cancer were selected and performed using fluorescence real-time RT-PCR. The expression level of β^+ ASV was 6.99 times higher in gastric cancer than that in precancerous lesions using the $2^{-\Delta\Delta CT}$ method.

Discussion

mRNA splicing is a fundamental biological process involved in the expression of genes, and about a third of human genes are thought to be alternatively spliced [15, 16]. Some alternatively spliced variants have been associated with a subset of human diseases [17–20]; however, the pathological importance of ASVs in most human diseases is still uncertain. Telomerase is activated in a variety of malignant tumors [6] and is thought to be necessary for cellular immortality and carcinogenesis. Telomerase activity was detected in gastritis patients with chronic atrophy, patients with intestinal metaplasia, dysplasia patients and gastric cancer patients. Telomerase is expressed during the early stage of gastric carcinogenesis [21]. Regulation of telomerase activity is a complex process involving transcriptional control, posttranslational modification, positively and negatively acting factors and alternative splicing [22, 23]. The core telomerase consists of two key components, the catalytic unit hTERT and an RNA template (hTER) [24]. Telomerase activity has been

shown to correlate well with the expression level of hTERT [25–27]. The hTERT gene includes 16 exons and 15 introns [28]. The hTERT protein contains one conserved reverse transcriptase motif A and seven reverse transcriptase motifs. hTERT mRNA has at least 13 alternative splicing sites, including 3 deletion sites (α , β , γ) and 10 insertion sites. In our study 3 deletion sites were selected to study the alternative splicing patterns in gastric carcinogenesis.

α -Deletion ASV and γ -deletion ASV result from in-frame deletions of exonic sequences in exon 6 and 11, respectively. The translation products of these two ASVs lost a small part of hTERT protein. Researchers speculated that these two ASVs appear to be inhibitors of telomerase activity [10, 29]. In our study, we found that the positive rates of α -deletion ASV and γ -deletion ASV had no statistical significance, and this result is consistent with a previous report [30]. We could not confirm that these two ASVs are inhibitors of telomerase activity. This may be because these two ASVs downregulated telomerase activity in a dose-dependent manner, but our study could not quantify them.

We observed that $\alpha^+\beta^+\gamma^+$ ASV transcript (unspliced ASV) was present in 40.0% of precancerous lesions and 94.7% of gastric cancer; however, it was absent in normal gastric mucosa. The difference was significant. The result was in agreement with that reported by Barclay et al. [30]. A previous study showed that expression of normal full-length hTERT correlates well with telomerase activity [31]. Telomerase activity was found to be higher in gastric

tumor samples than in normal tissue [32]. Barclay et al. [30] found there was a significant positive correlation between telomerase activity and hTERT mRNA expression in normal gastric mucosa, but there was no significant correlation between telomerase activity and levels of hTERT mRNA in gastric cancer. However, our study did not show the relationship of telomerase activity and full-length hTERT.

We observed that β -deletion ASV was widely expressed in normal gastric mucosa, precancerous lesions and gastric cancer. We also found that other ASVs ($\alpha\beta$ -deletion ASV, $\beta\gamma$ -deletion ASV, $\alpha\gamma$ -deletion ASV) were lower expressed in the three groups and the differences were nonsignificant. This may be because these ASVs lost many motifs of hTERT gene or because of the introduction of early stop codons. We did not detect $\alpha\beta\gamma$ -deletion ASV and this may be because it is unstable or easily degraded. Twelve cases of β^+ ASV (including 6 cases with precancerous lesions and 6 cases with gastric cancer specimens) were tested by real-time fluorescence quantitative RT-PCR. The results showed that the expression level of β^+ ASV was 6.99 times higher in gastric cancer than in precancerous lesions. The pathology reports of these specimens showed that 4/20 precancerous lesions were chronic atrophic gastritis with moderate or severe

atypical hyperplasia and 16/20 precancerous lesions were chronic atrophic gastritis with moderate to severe intestinal metaplasia. We found that β^+ ASV was expressed in all dysplasia specimens, but only a small number of intestinal metaplasia lesions (2/16) expressed β^+ ASV. Therefore, we assume that the expression of β^+ ASV may reflect the evolution of gastric cancer from intestinal metaplasia to dysplasia. The expression of β^+ ASV was higher in gastric cancer than in precancerous lesions. We guess that the increase in β^+ ASV may be involved in the carcinogenesis of precancerous lesions.

In conclusion, our study confirmed that hTERT alternative splicing patterns are different in gastric carcinogenesis. We assume that the increase in β^+ ASV may be involved in the carcinogenesis of precancerous lesions. The hTERT alternative splicing pattern is more complicated. In our study we only selected three splicing sites. If other new sites are found, further research will be necessary.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant No. 39770298).

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