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Telomerase activity could be used as a marker for neoplastic transformation in gastric adenocarcinoma: but it does not have a prognostic significance

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ABSTRACT. Telomerase activity is responsible for telomere maintenance and is believed to be crucial in most immortal cells and cancer cells; however, its clinicopathological significance in gastric cancer remains to be clarified. The aim of the present study was to assess whether malignant progression of gastric adenocarcinoma correlates with telomerase activity. We also investigated the correlation between telomerase activity and histopathological findings. We examined telomerase activity in tumor specimens and adjacent normal tissues from 43 patients with gastric adenocarcinoma. Telomerase activity was measured quantitatively by the TRAPeze Gel Based Telomerase Detection Kit. Approximately 98% of the tumor tissues were telomerase positive, but telo-

merase activity was detected not only in tumor tissues but also in normal gastric mucosa. Although telomerase activity was found to be higher in tumor samples than normal tissue for each subject, we could not find a general cut-off level for telomerase activity in gastric adenocarcinoma. In addition, telomerase activity was not correlated with tumor invasion, lymph node involvement and histological stage. Our results support the idea that telomerase reactivation is a common event in gastric adenocarcinoma and it is not related to histopathological parameters. Since it is difficult to set a cut-off level for this type of cancer, we suggest that the prognostic utility of telomerase assay has not yet reached the clinic in terms of predicting outcome for patients with gastric adenocarcinoma. For the assessment of gastric carcinoma, telomerase activity should be evaluated in both tumor and normal tissues, because normal gastric mucosa samples show appreciable telomerase activity.

Key words: Telomerase activity, Gastric adenocarcinoma, Tumor progression

INTRODUCTION

Telomeres are specialized structures at the end of all eukaryotic chromosomes and in human cells they are composed of repetitive TTAGGG sequences. In most human somatic cells, telomeres lose ~50-150 bp per cell division (Chan and Blackburn, 2004; Hahn, 2005). When the telomere length declines below a certain threshold, a replicative senescence is triggered. Replicative senescence may prevent chromosome instability, and is thought to limit the proliferative capacity of transformed cells (Muntoni and Reddel, 2005; Opitz, 2005). In most human cancers, the telomere barrier is bypassed through the activation of telomere maintenance mechanisms. Most commonly this is achieved by the activation of telomerase (Smogorzewska and de Lange, 2004; Muntoni and Reddel, 2005).

Telomerase is a specialized cellular reverse transcriptase that uses its RNA template to elongate the telomere by addition of G-rich telomeric repeats to the terminal 3' overhang (Chan and Blackburn, 2004; Dong et al., 2005). Telomerase is strongly suppressed in human somatic cells; however, robust telomerase activity (TA) is seen in ovaries, testes, and highly proliferative tissues as well as in cancer cells (Granger et al., 2002; Smogorzewska and de Lange, 2004).

Gastric adenocarcinoma is a significant world-wide health burden second only to lung tumors as a leading cause of cancer deaths (Jong et al., 1999; Rathi et al., 1999; Yokozaki et al., 2001). Although little is known about the cause and pathogenesis of gastric cancer, it is believed that enhanced understanding of the molecular basis of gastric cancer progression may lead to earlier diagnosis and an improvement of survival rate (Rathi et al., 1999). Telomerase activation is thought to be crucial in most immortal cells and cancer cells; however, its clinicopathologic significance in gastric cancer and the details of the mechanisms regulating TA remain to be

clarified (Yoo et al., 2003). In the present study our aim was to assess whether malignant progression of gastric adenocarcinoma correlates with TA. We also investigated the correlation between TA and histopathological findings.

MATERIAL AND METHODS

Tissue samples

Tumor samples were collected from 43 gastric cancer patients operated at Ankara University, School of Medicine, Department of Surgical Oncology between October 2004 and January 2006 after receiving their informed consent. Besides tissue samples for routine histopathological examination, a portion of tissue sample was used for telomerase assay. In addition, adjacent normal tissues were analyzed in parallel for detection of TA. Samples of normal mucosa were taken from areas near the surgical margins and far from the tumors that were macroscopically free of tumor invasion.

Telomerase assay

The tissue sections used for telomerase assay were immediately rinsed with PBS and were stored in a sterile RNase free microfuge tubes at -80°C until protein extraction. TA was examined by using the TRAPEZE® Gel Based Telomerase Detection Kit (Chemicon International) according to the manufacturer's instructions with slight modifications. Briefly, approximately 50-100 mg frozen tissue samples were minced on ice and extracts containing 110 ng protein were added to the TRAP reaction mixture containing 5 µL 10 X TRAP reaction buffer [200 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween20, and 10 mM EGTA], 1 µL 50X dNTP mix (2.5 mM each dATP, dTTP, dGTP, and dCTP), 1 µL TS primer (5'-AATC CGTCGAGCAGAGTT-3'), 1 µL TRAP primer mix (RP primer, K1 primer, TSK1 template), 2 U Taq DNA polymerase and dH_2O in a total volume of 50 µL. Reaction tubes were placed in a thermocycler (Thermolyne T1) and incubated at 30°C for 30 min. The reaction mixtures were subjected to 34 PCR cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 60 s. Protein extract from the telomerase positive cell pellet provided in the kit and the reaction mixture without tissue extract were used as positive and negative controls, respectively. In addition, TSR8 control template provided in the kit was used for the quantitation of the TA.

The PCR products were analyzed by electrophoresis at 350 V for 2.5 h on a 12% polyacrylamide gel. The gel was stained with SYBR Green I nucleic acid gel stain (Invitrogen) and visualized by Gel Logic 200 Image Analyzer. Images were analyzed with the Kodak 1D Software. The amount of TA was calculated using the following formula:

$$TA = \{[X/C] / [r/Cr]\} \times 50$$

where X is the intensity of the telomerase ladder of the test sample, C is the intensity of the internal standard in the test sample, r is the intensity of the TSR8 quantitation control, and Cr is the intensity of the internal standard in TSR8 quantitation control. After that, for all samples studied, TAs were calculated as per mg tissue.

Statistical analysis

Difference between TA in tumor and normal tissues was evaluated by the Wilcoxon signed ranks test. Differences among N0, N1 and N2 + N3 for TA in tumor and normal tissues and telomerase index (TI) were evaluated by Kruskal-Wallis variance analysis. Comparisons of TA in tumor and normal tissues and TI between two groups (T1 + T2 and T3 + T4; I and II + III) were analyzed by the Mann-Whitney U-test. P values less than 0.05 were considered to be statistically significant. Analyses were done with SPSS for Windows 11.5.

RESULTS

TA was detected not only in tumor tissues but also in normal gastric mucosa. TA in tumor tissues ranged from 1 to 2470, with a median value of 145 and in normal mucosa it ranged from 1 to 1266, with a median value of 44. The average TA (median) of tumor tissues was significantly higher than that of normal mucosa ($P < 0.001$). The ratio of the TA of gastric tumor tissues to that of corresponding normal mucosa was defined as the TI in order to exclude the TA of the background mucosa. The range of TI values was found to be between 0.3 and 223.1, with a median value of 2.

As can be seen, TA in tumor and normal tissues decreased with the depth of tumor invasion, where this was not statistically significant (Table 1, Figure 1). We did not find any correlation between lymph node involvement and TA. TA was higher in late stages (II and III) than early stage I; however, there was no significant correlation between TA and histological stage (Table 1).

TI was lower in T3 and T4 tumors than T1 and T2 tumors, and higher in late stage (II and III) tumors. However, all of these differences were not statistically significant. In addition, TI did not correlate with lymph node involvement (Table 1 and Figure 2).

DISCUSSION

TA has been reported in most tumor types (Klingelhutz, 1997; Granger et al., 2002), including gastric cancer (Jong et al., 1999; Yokozaki et al., 2001; Nowak et al., 2003; Yoo et al., 2003). In the present study, 42 of 43 patients (~98%) showed TA in their tumor tissues. Data in the literature and observations in this investigation indicate that telomerase reactivation may play a significant role in gastric carcinogenesis.

Clinicopathological significance of TA in human gastric cancer is controversial. Some investigators have indicated that the TA in tumor tissues correlates well with depth of invasion and tumor differentiation (Usselmann et al., 2001; Yoo et al., 2003). On the other hand, some investigators have shown no relation between clinical or histological factors and TA (Ahn et al., 1997; Heine et al., 1998; Jong et al., 1999; Furugori et al., 2000; Kameshima et al., 2000). In our study group, we also found no correlation between TA in tumor tissues and depth of tumor invasion, histological stage or lymph node involvement. This finding supports the idea that telomerase reactivation is a common event in gastric carcinogenesis.

In our present study, we found that 95% of the normal gastric mucosa specimens had detectable TA. Although TA is repressed in most somatic cells, it can be detected in highly proliferative tissues (Granger et al., 2002; Dong et al., 2005). The expression of telomerase

Table 1. Correlation between histopathological factors and telomerase activity/telomerase index.

Histopathological factors	N	Telomerase activity/mg tissue		Telomerase index
		Tumor tissue	Normal mucosa	
Tumor invasion				
T1 + T2	11	694.1 ± 841.3 168 (26-2470)	270.9 ± 386.2 105 (1-1266)	8.8 ± 17.5 2.2 (0.3-61)
T3 + T4	31	351.2 ± 546.7 130 (1-2454)	150.1 ± 220.1 43 (1-752)	10.9 ± 39.6 1.8 (0.4-223.1)
P value		0.35	0.41	0.37
Lymph node involvement				
N0	14	589.4 ± 872.1 134.5 (26-2470)	212.7 ± 354.9 45 (1-1266)	22.5 ± 59.8 2 (0.3-223.1)
N1	14	423.6 ± 597.9 108.5 (1-1803)	136.6 ± 200.8 50 (1-727)	3.9 ± 3.8 2.1 (0.4-12.6)
N2 + N3	14	310.1 ± 383.3 155.5 (42-1447)	196 ± 256.9 57.5 (6-752)	4.6 ± 5.3 1.7 (0.6-15.3)
P value		0.73	0.80	0.79
Histological stage				
I	21	325.8 ± 498.9 113 (1-1803)	126.9 ± 175.1 44 (1-727)	6.6 ± 13.2 1.8 (0.3-61)
II + III	22	552.2 ± 738.9 198.5 (21-2470)	227.2 ± 335.6 44.5 (6-1266)	14.2 ± 46.9 2 (0.6-223.1)
P value		0.24	0.61	0.40

Data are reported as means ± SD and as median with minimum and maximum values in parentheses.

components and TA may also be influenced by non-malignant pathological conditions such as *Helicobacter pylori* infection or chronic gastritis in the gastric mucosa (Kameshima et al., 2000; Nowak et al., 2003). It is pointed that in cases where the histological environment of the tumor is naturally telomerase expressing (as in this study), a positive result should be considered only when telomerase levels are higher than the matched control tissue (Granger et al., 2002). According to the results of our study, TA is significantly different than that in the normal mucosa. Therefore, gastric adenocarcinoma can be regarded as a highly telomerase positive cancer.

In order to exclude the TA of the background mucosa, the ratio of the TA of gastric tumor tissues to that of corresponding normal mucosa was defined as the TI proposed by Okusa et al. (2000). However, we did not find any correlation between TI values and the histological parameters.

Both TA in tumor/normal tissues and TI values showed great inter-individual variability. Therefore, it was difficult to set a general cut-off level of TA for gastric adenocarcinoma. This

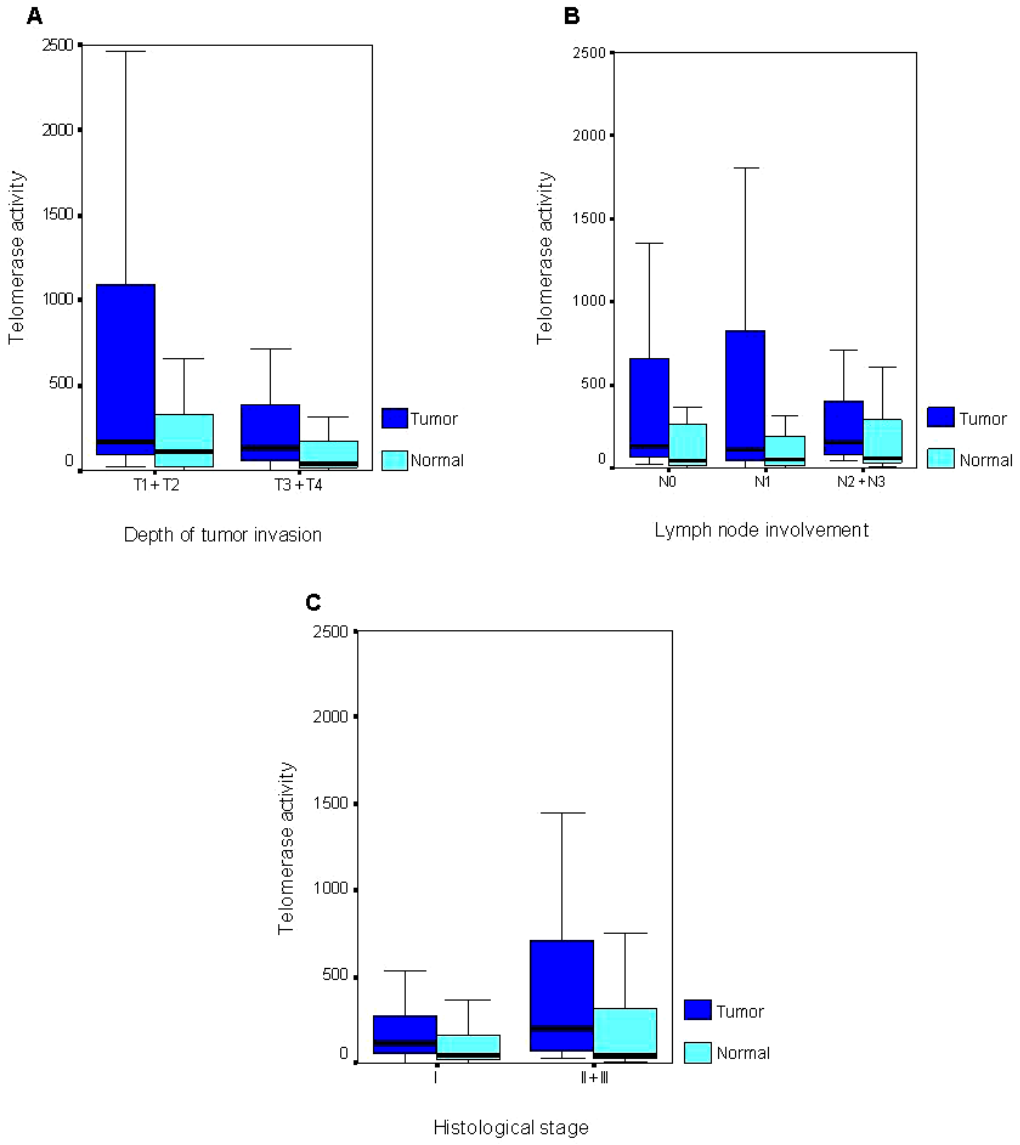

Figure 1. Correlation between histopathological factors (A: Depth of tumor invasion, B: Lymph node involvement, C: Histological stage) and telomerase activity. Comparisons of telomerase activity in tumor and normal tissues between groups with different depth of tumor invasion, and histological stage were analyzed by the Mann-Whitney U-test. Kruskal-Wallis variance analysis was used for comparison of telomerase activities in N0, N1 and N2 + N3 groups.

finding supports the study by Nakamura et al. (1999) who proposed that the difficulty in setting a cut-off level was probably due to the intrinsic characteristics of the gastrointestinal tissues, since noncancerous parts, including cryptic epithelium and possibly lymphocytes, were shown to have substantial levels of TA.

In contrast to other studies, it is interesting that tumors with deeper invasion had lower TA compared with T1 and T2 tumors, although this difference was not statistically significant. It

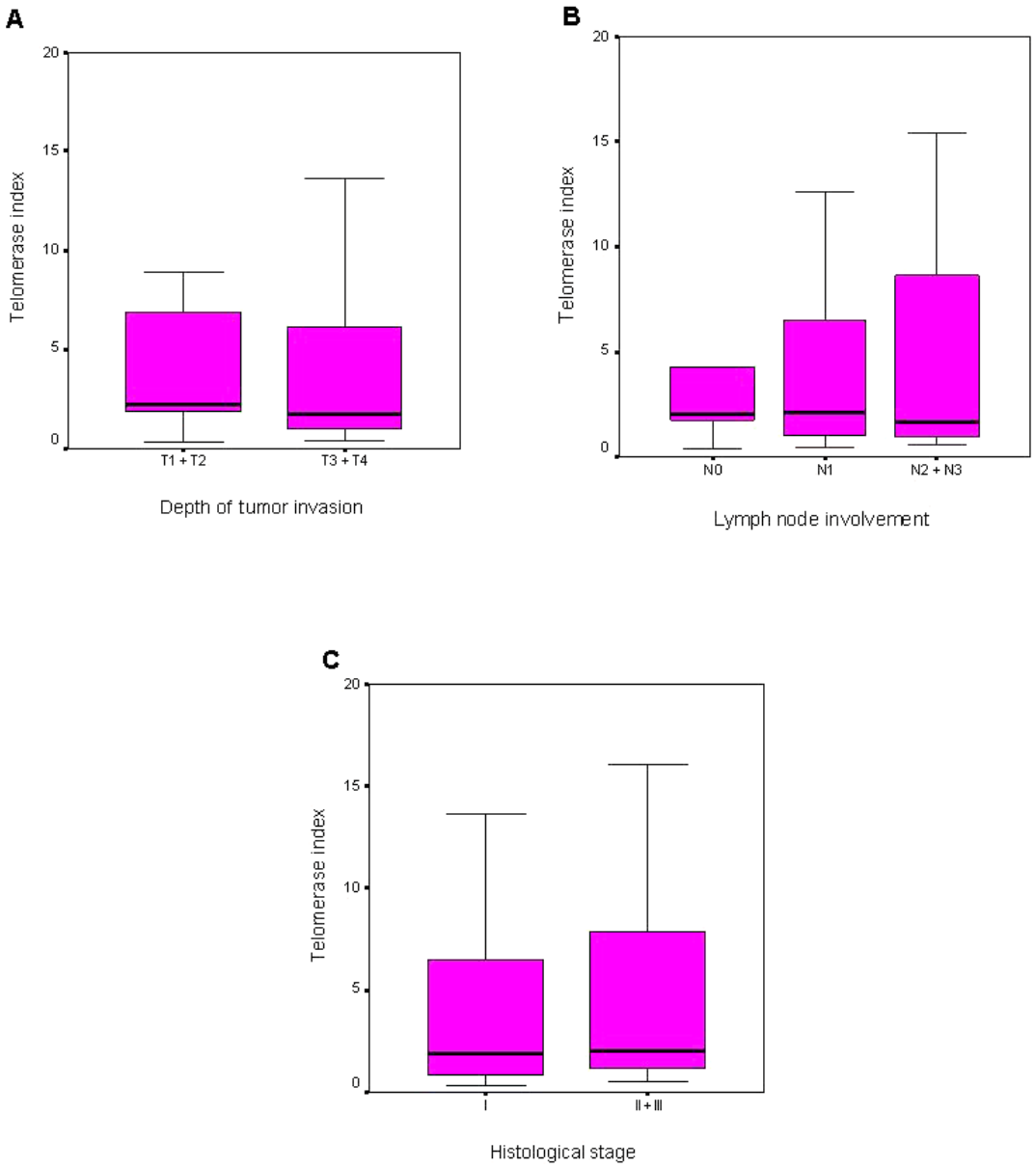

Figure 2. Correlation between histopathological factors (A: Depth of tumor invasion, B: Lymph node involvement, C: Histological stage) and telomerase index. Comparisons of telomerase index between groups with different depth of tumor invasion, and histological stage were analyzed by the Mann-Whitney U-test. Kruskal-Wallis variance analysis was used for comparison of telomerase index in N0, N1 and N2 + N3 groups.

is known that lymphocytes contribute to the TA of the tissues that they infiltrate. It has been reported that lymphocytic infiltration is negatively correlated with the depth of tumor invasion in gastric cancer (Ishigami et al., 2000a,b). Therefore, we suggest that the lower TA in T3 and T4 tumors may be caused by the decreased lymphocyte infiltration in these tumors.

Another reason for the decreased TA in T3 and T4 tumors could be related to telomere maintenance mechanisms other than the telomerase. Tumor cells with no TA have acquired telomerase-independent mechanism for lengthening telomeres, namely ALT (alternative lengthening of telomeres) (Granger et al., 2002; Muntoni and Reddel, 2005). Evidence indicates that some tumors possess only TA or only an ALT mechanism and some have both. The types of tumors and tumor cell lines in which ALT has been observed include osteosarcoma, soft tissue sarcoma, glioblastoma multiforme, renal cell carcinoma, non-small cell carcinoma of the lung, and ovarian cancer (Reddel and Bryan, 2003; Stewart, 2005). However, more extensive surveys need to be done to identify the other tumor types in which ALT are common. Data obtained from the studies on the relationship between ALT and tumor aggressiveness are controversial. Therefore, many more studies are necessary to clarify the effects of ALT on tumor prognosis. However, there is evidence showing that patients with telomerase positive-ALT positive tumors have the worst prognosis (Reddel and Bryan, 2003; Stewart, 2005).

Kim et al. (2002) have reported an Adriamycin-resistant stomach cancer cell line with decreased TA, and they propose a possible ALT-like mechanism for telomere maintenance in these cells. This finding supports the possibility that gastric cancer cells may have ALT or ALT-like mechanisms to maintain their telomeres. We propose that T3 and T4 tumors surveyed in this study might have acquired an ALT or similar mechanisms for maintaining their telomeres in addition to their TA, and this might have resulted in decreased TA and increased invasion capacity in these tumors. However, this possibility should be evaluated in more detail.

Decrease in TA caused by either lower lymphocyte infiltration or ALT mechanism may mask the TA changes in late stage tumors (stages II and III). In addition, since late stage tumors could be different in respect to their degree of invasion, it is difficult to find a general cut-off TA level for tumor invasion. In order to find a real correlation, many more samples with the same tumor invasion and histological stage properties should be compared in terms of their TAs.

In conclusion, our results support the idea that telomerase reactivation is a common event in gastric adenocarcinoma and it is not related to the histopathological parameters. Since it is difficult to set a general cut-off level for this type of cancer, the prognostic utility of telomerase assay has not yet reached the clinic in terms of predicting outcome for patients with gastric adenocarcinoma. According to the results of our current study, it is clear that normal gastric mucosa samples show appreciable TA. Therefore, for the assessment of gastric carcinoma, TA should be evaluated in both tumor and normal tissues. Moreover, further investigation is needed to clarify the role of TA in gastric carcinogenesis, and the mechanisms other than the telomerase should not be neglected.

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