Pergamon

European Journal of Cancer Vol. 33, No. 8, pp. 1309–1313, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00113-5

Original Paper

Telomerase Activity and its Clinicopathological Significance in Gastric Cancer

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In order to assess the role of telomerase in development of malignant gastric cancer, we measured the telomerase activity in gastric cancer tissues and normal tissues obtained from 95 patients by employing recently developed sensitive PCR (polymerase chain reaction)-based telomerase assay (telomeric repeat amplification protocol, TRAP). We also investigated how telomerase activity related to other clinicopathological findings including DNA ploidy and *K-RAS* gene point mutation. The telomerase activity was present in 85 of the 95 gastric cancer tissues, whereas we detected no telomerase activity in any normal tissue. The incidence of telomerase activity in gastric cancer tissues was not correlated to age, sex, tumour stage, histological grade, DNA ploidy or *K-RAS* mutation. Disease-free or overall survival of patients having tumours with detectable telomerase activity was not significantly different from that of those without telomerase activity. These findings suggest that telomerase may play a key role in the establishment and progression of the gastric cancer and further studies will be needed to elucidate the biological role of telomerase in gastric cancer. © **1997 Published by Elsevier Science Ltd.**

Key words: telomerase, gastric cancer, DNA flow cytometry, *K-RAS* oncogene

Eur J Cancer, Vol. 33, No. 8, pp. 1309–1313, 1997

INTRODUCTION

THE MALIGNANT phenotype of human cancer results from multiple independent mutations that activate proto-oncogenes or inactivate tumour suppressor genes [1]. Although several authors have reported findings in gastric cancer that support this concept [2], the molecular mechanism of gastric carcinogenesis has not been fully explored.

Human cancers are characterised by malignant transformation and immortalisation [3]. However, difficulty in establishing cell lines from cancer tissue samples has led many investigators to question whether cancer cells *in vivo* are immortal or not [4].

Presented at 87th AACR meeting in Washington DC, U.S.A.
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Received 17 Sep. 1996; revised 10 Jan. 1997; accepted 30 Jan. 1997.

It has been shown that telomere DNA shortens at chromosome ends with division, both *in vivo* and *in vitro*, of human somatic cells unless the termini are extended specifically by telomerase [5–7]. Telomerase is a ribonucleoprotein, RNA-dependent DNA polymerase, and acts as a reverse transcriptase-like enzyme which maintains telomere length by adding telomeric repeat units of TTAGGG to the telomere end [8, 9]. It has been suggested that deregulation of telomerase may participate in cellular immortality and oncogenesis. Cells with indefinite replicative potential such as germline cells, almost all tumour cell lines and many cancer tissues express telomerase activity [10–12], while normal human somatic cells show low or undetectable telomerase activity and progressively lose their telomeric sequences both with senescence *in vitro* and with normal *in vivo* ageing [5, 13–15]. Therefore, telomerase activation may be directly involved in cell immortalisation and telomere maintenance.

With conventional methods, extraction of telomerase required at least 10^7–10^8 cells and efficiency varies between cell types [16, 17]. The recently developed sensitive polymerase chain reaction (PCR)-based telomerase assay (telometric repeat amplification protocol, TRAP) makes it possible to detect the enzyme in a variety of human tumours and tumour-derived cell lines [10]. Using this new method, we examined telomerase activity in human gastric cancer tissues and normal tissues to determine whether malignant progression of gastric cancer may correlate with expression of telomerase. We also investigated the correlation between telomerase positivity and other clinicopathological findings.

MATERIALS AND METHODS

Detergent extracts from tissues

Gastric cancer tissues and normal tissues were obtained from each of 95 patients undergoing surgery (Asan Medical Center, Seoul, Korea; 1990–1992). Each tissue sample, consisting of 100 mg of frozen tissue (−70°C), was washed in ice-cold buffer [10 mM Hepes-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), and then homogenised in a Kontes tube with matching pestles rotated at 450 rpm with 200 µl of ice-cold lysis buffer [10 mM tris-HCl (ph 7.5), 1mM $MgCl_2$, 1 mM EGTA 0.1 mM phenylmethylsulphonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol]. The lysate was incubated for 30 min on ice and then centrifuged for 30 min in a microcentrifuge (16 000*g*, 4°C). Human 293 kidney cells, which would serve as a positive control, were collected from 100 mm culture dishes, washed twice with ice-cold phosphate-buffered saline (PBS), once with ice-cold wash buffer, and centrifuged at 3000*g* for 5 min at 4°C. The pellets were resuspended in 20 µl of ice-cold lysis buffer per 10^6 cells, and processed in the same way as the tissue extracts. The supernatant was quickly frozen in liquid nitrogen and stored at −70°C.

Amplification of telomeric repeat

Telomerase activity was assayed by using the TRAP method [10]. In brief, 0.1 µg of lyophilising CX primer (5′-{CCCTTA}$_3$CCCTAA-3′) was placed in a PCR tube and sealed with 100 µl of wax (Ampliwax, Perkin–Elmer Cetus Corp., Foster City, California, U.S.A.). After the wax was allowed to solidify at room temperature, the tubes were stored at 4°C. Fifty microliters of TRAP reaction mixture [20 mM tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 50 µM deoxynucleoside triphosphate, 0.1 µg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 2 U of Taq polymerase (Promega, Madison, Wisconsin, U.S.A.) and 2–8 µl of a CHAPS cell extract (6 µg of protein) were layered above the wax. Tubes were held for 30 min at room temperature for telomerase to extend oligonucleotide TS, then transferred to the thermal cycler (Robocycler 40; Stratagene, La Jolla, California, U.S.A.) for 40 rounds at 94°C for 30 s, 50°C for 30 s and 72°C for 45 s. The resulting solution was analysed by electrophoresis in 1 × tris-borate EDTA (TBE) buffer on 15% polyacrylamide nondenaturing gels. A silver staining method was used for visualising the PCR products after electrophoresis.

DNA preparation and K-RAS *oncogene point mutation*

Genomic DNA was isolated by using a standard procedure with proteinase K incubation and repeated phenol-chloroform extractions. To detect the *K-RAS* codon 12 mutation, the double PCR method was used as previously described [18, 19]. This method is based on the PCR with mismatched primers creating a new diagnostic restriction site in the normal, wild-type sequence, and thus mutant alleles are not cleaved by restriction enzymes. Further control mismatches were introduced into the above primers to discriminate mutants from uncleaved residual PCR products, the result of incomplete digestion. The primers used were: 5′-ACTGAATATAAACTTGTGGTAGTTGGACCT-3′ and 5′-TCAAAGAATGGTCCTGGACC-3′ (first PCR step) or 5′-ATTAAAACAAGTTTACCTC-3′ (second PCR step). Details of these modifications are described elsewhere [19]. The A549 human lung carcinoma cell line served as a positive control for the codon 12 mutation. DNAs were amplified in a thermal cycler using Taq polymerase. PCR products were digested with BstNI restriction enzyme and analysed by using 2.5% agarose gel electrophoresis. The PCR products showing *K-RAS* 12 mutation were sequenced using Sequenase (U.S. Biochemical, Cleveland, Ohio, U.S.A.) according to the method of Sanger and associates [20].

DNA flow cytometry

DNA ploidy and S-phase fraction were measured by DNA flow cytometry as previously described [21, 22].

Statistical analysis

Frequencies of telomerase activity were compared with various clinical characteristics and pathological variables using Fisher's exact test. Wilcoxon's rank sum test was employed to analyse S-phase fractions. Survival curves were obtained using the Kaplan–Meier method and compared with the frequencies of the telomerase activity using the generalised Wilcoxon test.

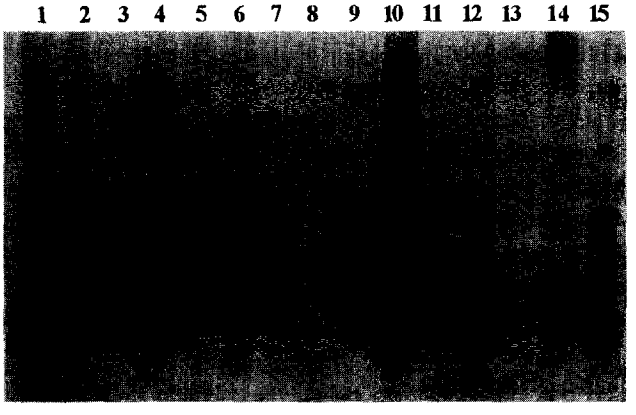

Figure 1. Telomerase activity in gastric cancer tissues. Most of the gastric cancer tissues revealed telomerase activity which appeared as characteristic 6 base pair laddering, whereas some had no telomerase activity (lanes 5, 8). Human 293 kidney cells served as a positive control (lane 1).

Table 1. Patient characteristics in gastric cancer

	Telomerase activity		
	Positive (n = 85)	Negative (n = 10)	P value
Age (years)			
<60 (n = 60)	52	8	0.209
⩾60 (n = 35)	33	2	
Sex			
Male (n = 57)	51	6	0.639
Female (n = 38)	34	4	
Tumour location			
Upper (n = 6)	6	0	0.753
Middle (n = 20)	19	1	
Lower (n = 67)	58	9	
Diffuse (n = 2)	2	0	
TNM stage			
I (n = 7)	7	0	0.984
II (n = 19)	17	2	
IIIa (n = 19)	17	2	
IIIb (n = 35)	30	5	
IV (n = 15)	14	1	
Histological grade			
Well differentiated (n = 4)	4	0	0.315
Moderately well differentiated (n = 18)	18	0	
Poorly differentiated (n = 66)	56	10	
Undifferentiated (n = 7)	7	0	
Lymph node metastasis			
N0 (n = 20)	17	3	0.827
N1 (n = 32)	29	3	
N2 (n = 43)	39	4	

RESULTS

Telomerase activity and clinicopathological data in gastric cancers

Telomerase activity was positive in 85 of 95 gastric cancer tissues (89%). Fourteen representative results are shown in Figure 1. The positivity rate of telomerase was 100% (7 of 7) in stage I; 89% (17 of 19) in stage II; 89% (17 of 19) in stage IIIa; 86% (30 of 35) in stage IIIb; and 93% (14 of 15) in stage IV gastric cancer (Table 1). The expression of telomerase in gastric cancers was not correlated to age, sex, tumour location, tumour stage, histological grade or presence of lymph node metastasis (Table 1). Normal tissues extracted from each gastric cancer patient did not show any telomerase activity as shown in Figure 2.

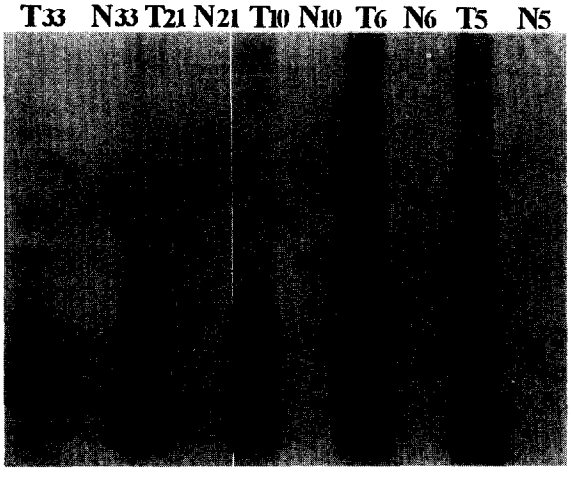

Figure 2. Representative results of telomerase activity in gastric cancer and normal tissues. Most of the gastric cancer tissues (T) showed telomerase activity, whereas it was not detected in corresponding normal tissues (N).

Table 2. Telomerase activity and DNA ploidy in gastric cancer

	Telomerase activity	
	Positive (n = 84)	Negative (n = 10)
DNA ploidy		
Diploid (n = 44)	40	4
Aneuploid (n = 50)	44	6

Telomerase activity and DNA flow cytometry

All tumour samples except one were examined to investigate the cellular DNA content of tumour cells by means of the flow cytometry. Of 94 tumours, 44 (47%) were diploid and 50 were aneuploid. Forty of the 44 (91%) diploid and 44 of 50 (88%) aneuploid tumours showed telomerase activity, but there was no significant correlation between DNA ploidy and telomerase activity (Table 2).

Telomerase activity and K-RAS *mutation*

Only 7 of the 95 samples (7%) showed *K-RAS* codon 12 mutation. All the patients with *K-RAS* mutation showed telomerase activity, although this association was not statistically significant (Table 3).

Disease-free and overall survival

Disease-free survival (DFS) and overall survival (OS) rates were not significantly different among patients having gastric cancers with or without telomerase activity (Figure 3 and Table 4).

DISCUSSION

Telomerase activity has been detected in a wide variety of human tumours and tumour-derived cell lines, whereas it is not found either in normal cells *in vitro* or normal somatic tissues *in vivo* [10, 11]. In our study, telomerase activity was positive in 85 of 95 (89%) gastric cancer tissues whereas normal tissues did not show any telomerase activity. Such high positive rates were also demonstrated in our previous reports, one for head and neck cancer (89%) [23], and the other for cervical cancer (89%) [24]. These findings suggest that telomerase activation may play a significant role in the establishment and progression of cancer of a variety of histological types.

All the seven early-stage gastric tumours (stage I) and 78 of 88 advanced-stage gastric tumours (stage II, III and IV) showed telomerase activity. In carcinoma *in situ* (CIS) and even in dysplasia of cervical epithelium, the majority displayed telomerase activity [24]. These results indicate that telomerase activation may be present not only in late but also in early stages of cancers.

Table 3. Telomerase activity and K-RAS codon 12 mutation in gastric cancer

	Telomerase activity	
	Positive (n = 85)	Negative (n = 10)
K-RAS mutation		
Positive (n = 7)	7	0
Negative (n = 88)	78	10

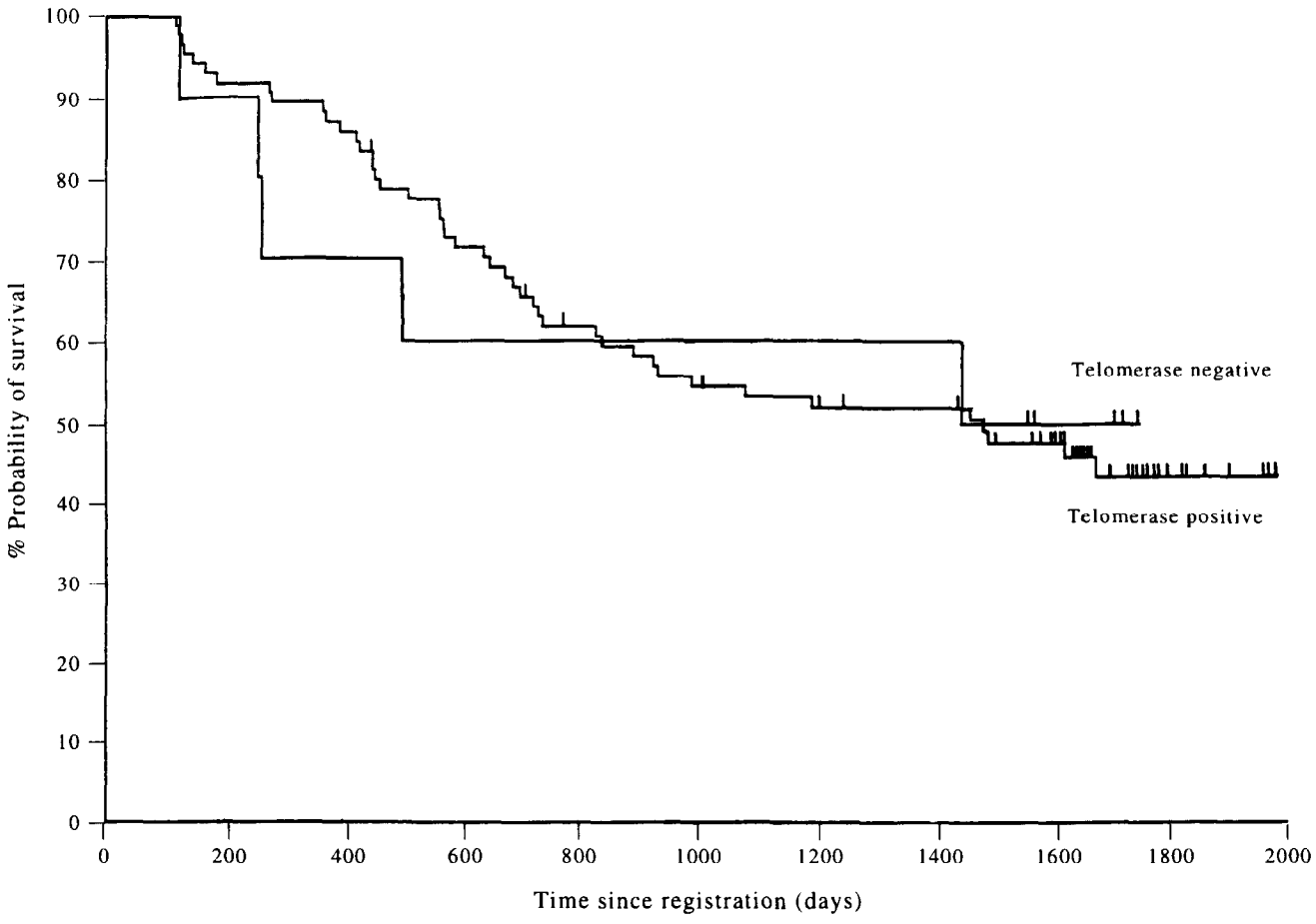

Figure 3. Overall survival for patients with telomerase positive (n = 85) or telomerase negative (n = 10) tumours.

Telomerase activity in gastric cancer was not correlated to age, sex, tumour location, tumour size, tumour stage, histological grade or lymph node metastasis. We also found that the DFS and OS among patients of gastric cancers with or without telomerase activity did not show significant difference. Our data are in contrast to those of Hiyama and associates [25], who reported that telomerase activity was present mostly in large tumours or those of advanced stage, including metastatic tumours. Furthermore, the survival rate of tumour patients having detectable telomerase activity was significantly lower than that of patients without telomerase activity. These contradictory results need further investigation.

Some of the gastric cancer tissues (10 of 95) did not show any telomerase activity in three independent experiments. We re-assayed three times with these samples including internal control primers (TRAP-eze Telomerase Detection Kit, Oncor) to exclude the possibility of existence of a telomerase inhibitor, but all the initially negative samples did not contain a telomerase inhibitor (data not shown). The absence of telomerase activity may be due to degradation of the essential telomerase-templating RNA [26].

Table 4. Disease-free and overall survival rates in patients of gastric cancers with or without telomerase activity

	Telomerase activity		
	Positive (%)	Negative (%)	*P* value*
Relapse	37/74 (50)	4/9 (44)	0.987
Death	40/85 (47)	5/10 (50)	0.769

*Wilcoxon text

The prognostic significance of DNA aneuploidy has been established in many human cancers [26–29]. However, DNA aneuploidy may not be so significant in gastric cancer [21, 31]. We found that 50 of 94 tissue samples (53%) showed aneuploidy and the majority of aneuploid tumours (88%) had telomerase activity.

The incidence of *K-RAS* point mutation at codon 12 in gastric cancer is relatively low [19, 32] and its prognostic significance is still controversial [19, 32, 33]. Although all seven cases with *K-RAS* mutation also showed telomerase activity, many tumours without *K-RAS* mutation showed telomerase activity, indicating that there is no apparent correlation between *K-RAS* codon 12 point mutation and telomerase activity.

In conclusion, the present study demonstrated that telomerase activity is high in gastric cancers, irrespective of the tumour size, location, stage, histological grade, DNA ploidy or *K-RAS* codon 12 mutation. These findings indicated that telomerase may be involved in malignant transformation and in the progression of gastric cancers. Further studies are necessary in order to understand fully the biological role of telomerase in gastric cancer.

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Acknowledgements—Supported in part by a grant from the Research Fund of Hanyang University in 1995.