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Gene Expression for Suppressors of Telomerase Activity (Telomeric-repeat Binding Factors) in Breast Cancer

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Mechanisms regulating telomerase activity and telomere length remain incompletely understood in human breast cancer. We therefore studied gene expression for telomeric-repeat binding factors (TRFs) in relation to telomerase activity, telomere length, and clinicopathologic factors in human breast cancer. Telomerase activity was detected in 65.8% of 38 breast cancers, but none of 16 noncancerous samples. Terminal restriction fragments were longer in noncancerous than in cancerous tissues, but not significantly. Among 8 patients with both cancer and paired noncancerous tissue available for terminal restriction fragments length assay, terminal restriction fragments were shorter in cancers than in paired noncancerous samples in all but one. Significantly more mRNA encoding TRF1 and 2 was detected in noncancerous than in cancer tissues. Additionally, expression of TRF1 and 2 mRNA was significantly higher in cancers without detectable telomerase activity than in cancers showing activity. Expression of these genes tended to show a negative correlation with terminal restriction fragments length, but this was not statistically significant. No correlation was seen between TRF1 or 2 mRNA expression, and clinicopathologic factors except for TRF1 with respect to tumor size and progesterone receptor status. In addition to reactivation of telomerase activity, escape from negative regulation of this activity is needed to maintain telomere length during cell proliferation in breast cancer. Genes encoding telomerase inhibitors might be of value in gene therapy against human breast cancer.

Key words: Telomeric-repeat binding factors — Breast cancer — Telomere length — Telomerase activity — Gene expression

Several molecular changes in breast cancer have been demonstrated, including overexpression of HER2/neu (also known as c-erbB-2) and p53, reduced expression of BRCA1, and DNA aneuploidy.[1–6)]

Telomerase is an enzyme that replaces repeating TTAGGG sequences on ends of chromosomes that otherwise would be lost with successive cell divisions.[7)] Accordingly, telomerase activity is linked closely to attainment of cellular immortality, a step in carcinogenesis, while lack of such activity contributes to cellular senescence.[8, 9)] Telomerase activation has been detected in more than 85% of cases in studies of cancers of the stomach, colon, liver, breast, bladder, and prostate, as well as in lymphomas and leukemias.[10–14)] In contrast, telomerase activity usually is repressed in normal somatic tissues except in certain self-renewing tissues with high regenerative potential. Manipulation of telomerase has generated considerable excitement as a potential anticancer strategy.

Most cancers have short terminal restriction fragments, as the cells have undergone many divisions and telomere length is not regulated by telomerase alone. Two human telomeric-repeat binding factor proteins (TRFs) recently have been cloned. TRF1 is considered to inhibit the action of telomerase at the telomeric region, while TRF2 is believed to prevent fusion of chromosome ends; *in vitro*, TRF2 remodels linear telomeric DNA into large duplex loops.[15–19)] However, details of mechanisms regulating telomerase activity and telomere length still are poorly understood in human breast cancer.

Using a quantitative TaqMan reverse transcription-polymerase chain reaction (RT-PCR) for mRNAs encoding TRF1and TRF2, we studied gene expression for TRFs in relation to telomerase activity, telomere length, and clinicopathologic factors in human breast cancer.

MATERIALS AND METHODS

Tissue samples Samples of breast cancer and paired samples of noncancerous tissues from the same resected breast were obtained from 38 patients following surgery at Sapporo Medical University Hospital and Sapporo Breast Surgical Clinic between August 1996 and August 2000 (Table I). All tissue samples from resected specimens were used in this study after informed consent was obtained from the patients or guardians. Samples dissected from these speci-

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mens were immediately frozen. Histologic diagnosis was determined in formalin-fixed tissues immediately adjacent to the frozen samples used for mRNA assay. Clinicopathologic factors were evaluated according to the criteria of the Japanese Society of Breast Cancer.[20]

Telomerase assay Tissue samples were homogenized and lysed as described previously.[8] After incubation on ice for 30 min, lysates were centrifuged at 16 000*g* for 30 min at 4°C. Supernatants were collected and their protein concentrations were measured by a Gene Quant DNA/RNA Calculator (Pharmacia, Uppsala, Sweden); lysates contained 0.06 μg of cellular protein. Telomerase activity was measured using a PCR-based Telomeric Repeat Amplification Protocol (TRAP)-eze Telomerase Detection Kit (Oncor, Gaithersburg, MD) according to the method described by Kim *et al.* previously.[21] Briefly, each TRAP reaction mixture was incubated at 30°C for 30 min followed by 27 cycles of 94°C for 30 s and 60°C for 30 s in a thermal cycler (model 9600; Perkin-Elmer, Foster City, CA). Fifteen microliters of the PCR product was electrophoresed in 0.5× Tris-borate EDTA buffer on 12.5% polyacrylamide nondenaturing gels. Gels were dried and processed for autoradiography, exposing sensitive New A film (Konica, Tokyo) at −80°C for 3 h. Signal intensity on exposed films was measured using a Personal Densitometer model SI (Molecular Dynamics, Sunnyvale, CA). The experimental sample was incubated at 85°C for 10 min prior to the TRAP assay to inactivate telomerase and serve as a negative control, and a cell extract of known telomerase content provided in the kit served as a positive control. Semiquantitative analysis to estimate relative telomerase activity was accomplished by performing the TRAP assay with a TSR8 control template provided in the kit in place of sample extract. Telomerase activity was calculated in units of total product generated (TPG) using a formula as described previously[21]: $\mathrm{TPG}=\{[(x-x_0)/c]/[(r-r_0)/c_R]\}\times 100$, where telomerase products from non-heat-treated sample extract are x, telomerase products from a heat-treated sample extract are x_0, a non-heat-treated sample extract as an internal control is c, telomerase product TSR8 quantification control is r, telomerase products from the lysis buffer only are r_0, and the internal control for TSR8 quantification is c_R.

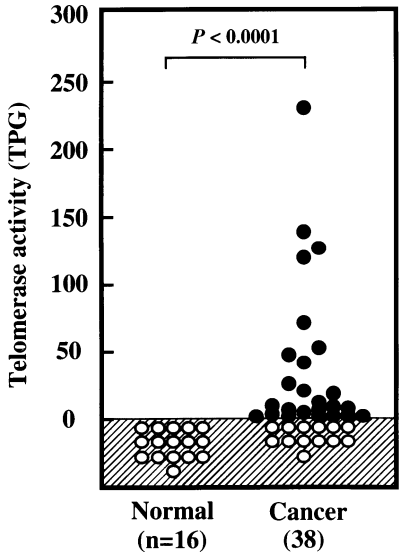

Fig. 1. Telomerase activity in breast cancer and noncancerous tissues. Samples with telomerase activity are indicated as (●), and those without telomerase activity as (○). TPG, total product generated.

Terminal restriction fragments Genomic DNA was extracted from tissues using SepaGene (Sanko Pure Chemical, Tokyo). Terminal restriction fragments were measured using a Telo*TAGGG* Telomere length assay kit according to the manufacturer's instructions (Roche Molecular Biotech, Mannheim, Germany). One to two micrograms of DNA was digested with *Hinf*I and *Rsa*I at 37°C for 4 h and electrophoresed on 1% agarose gels. The digested DNAs then were blotted onto nylon membranes (Boehringer, Mannheim, Germany) and hybridized with a digoxigenin (Dig)-labeled telomere specific hybridization probe. Hybridized blots were washed, then anti-Dig antibody conjugated with alkaline phosphatase was applied and developed with CDP-Star (Roche Molecular Biotech). We estimated the mean length of terminal restriction fragments at the most dense area of the hybridization signal as determined using a Personal Densitometer (model SI).

Quantitative RT-PCR assays for TRF1 mRNA and TRF2 mRNA SepaGene RV-R (Sanko Pure Chemical) was used to extract total RNA from tissues, and this extract was assayed for RNA with a Gene Quant DNA/RNA Calculator (Pharmacia). For quantitative RT-PCR, fluorescent hybridization probes, TaqMan PCR Core Reagents Kit with AmpliTaq Gold (Perkin-Elmer) were used with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Expression of TRF1 and TRF2 mRNA was quantified by methods previously reported.[22–24] Twenty-five nanograms of total RNA from samples was used for one step RT-PCR. Conditions of one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). Data for TRF1 mRNA and TRF2 mRNA were normalized to data for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical analysis Analysis of variance (Mann-Whitney) was used for comparisons of TRF1 and TRF2 mRNA expression and telomere length in clinical samples.

RESULTS

Telomerase activity and telomere length Telomerase activity was examined in 38 breast cancer samples and 16 noncancerous breast samples. Telomerase activity was detected in 65.8% (25/38) of breast cancers, but not in any noncancerous sample (Fig. 1). When the length of terminal restriction fragments was examined in 35 breast cancer tissues and 13 noncancerous breast tissues, these fragments were longer in noncancerous tissues (mean±SD and range; 7.1±0.9 kb and 5.1 to 8.4 kb) than in breast cancers (6.4±1.3 kb and 4.4 to 10.2 kb), including 13 tumors without detectable telomerase activity; however, the difference in length fell short of significance (Fig. 2A). In addition, 8

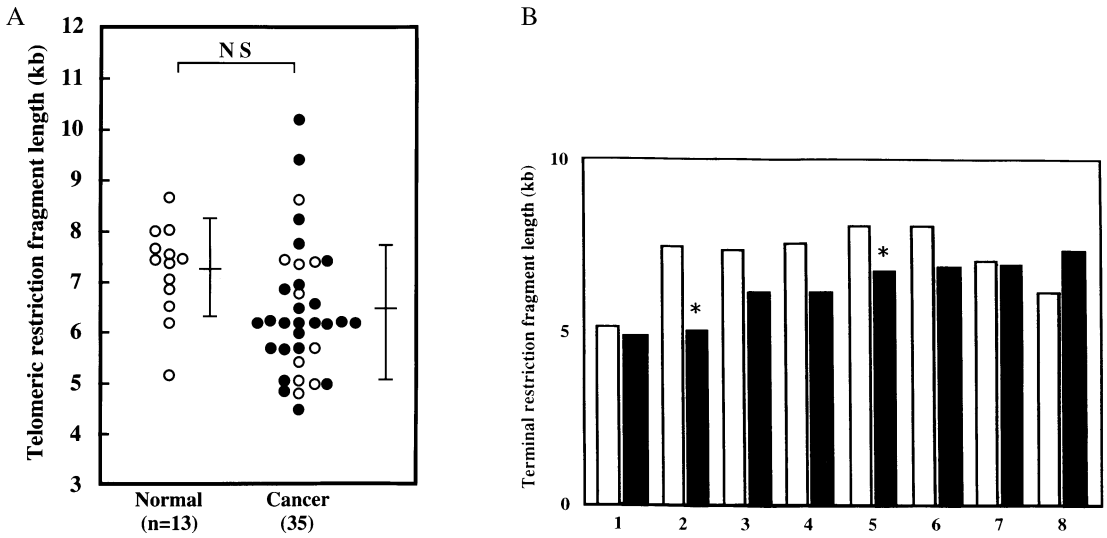

Fig. 2. Length of terminal restriction fragments. A, overall comparison between breast cancer and noncancerous tissues. Samples with detectable telomerase activity are indicated as (●), and those without detectable telomerase activity as (○). Bars indicated mean±SE. B, terminal restriction fragments in breast cancer (■) and paired noncancerous tissues (□). The symbol * indicates samples without detectable telomerase activity. Sample numbers 1 to 8 each represent a different patient. NS, not significant; kb, kilobase pairs.

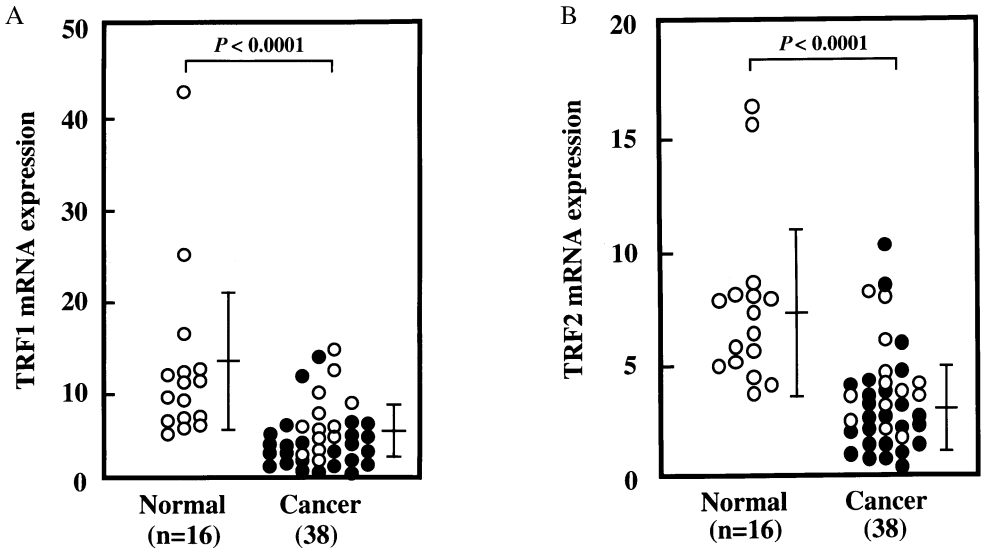

Fig. 3. TRF1 and TRF2 mRNA expression in breast cancer and noncancerous tissues. A, TRF1. B, TRF2. Samples with detectable telomerase activity are indicated as (●), and those without detectable telomerase activity as (○). Bars indicate mean±SE. TRF, telomeric-repeat binding factor.

Table I. Clinicopathologic Factors and Expression of TRF1 and TRF2

Characteristics		*n*	TRF1		TRF2	
			expression	*P*	expression	*P*
Menopausal status	pre	22	5.68±4.3	0.836	3.77±2.7	0.574
	post	16	5.06±3.4		3.19±1.6	
Clinical stage	I	6	8.88±5.6	0.055	5.32±3.6	0.093
	II·III	32	4.77±3.2		3.19±1.8	
Histologic type	Invasive lobular	3	7.18±3.4	n.c.	3.42±0.7	n.c.
	Mucinous	2	1.64±1.4		0.61±0.1	
	Papillotubular	17	6.12±4.4		4.33±1.2	
	Schirrhous	13	3.59±1.6		2.48±2.9	
	Solid tubular	3	6.86±6.6		3.25±3.0	
Tumor size (cm)	<2	14	7.27±4.5	0.018	4.54±2.9	0.085
	≥2	24	4.34±3.1		2.93±1.7	
Lymph node metastasis	absent	16	5.88±3.9	0.294	3.65±2.3	0.701
	present	22	5.08±3.9		3.43±2.3	
Estrogen receptor[a)]	negative	20	5.02±3.5	0.591	3.13±2.2	0.386
	positive	11	5.31±3.1		3.67±1.9	
Progesterone receptor[b)]	negative	11	3.60±1.8	0.043	2.34±1.2	0.091
	positive	18	6.19±3.6		4.12±2.3	

a) Cutoff value for estrogen receptor, <13 fmol/mg.
b) Cutoff value for progesterone receptor, <10 fmol/mg.
TRF, telomeric-repeat binding factor protein; n.c., not calculated.

patients had paired breast cancer and noncancerous samples available for length of terminal restriction fragments assay; in all but 1 patient, terminal restriction fragments were shorter in cancers than in paired noncancerous samples (Fig. 2B). No significant correlation was evident between length of terminal restriction fragments and telomerase activity in the 25 breast cancer tissues with detectable telomerase activity ($y=9.05x-18.79$, $r=0.209$).

Expression of mRNA for TRFs TRF-encoding mRNAs were examined in 38 breast cancers and 16 noncancerous breast samples. Expression of TRF1 mRNA and TRF2 mRNA was significantly higher in noncancerous tissues than in breast cancer tissues (Fig. 3). In addition, expression of TRF1 mRNA ($P<0.001$) and TRF2 mRNA ($P<0.016$) was higher in breast cancer tissues without detectable telomerase activity ($n=13$) than in breast cancer tissues with detectable telomerase activity ($n=25$).

In breast cancer tissues with detectable telomerase activity, we examined the relationship between gene expression for TRFs and length of terminal restriction fragments. A tendency toward negative correlation was seen between expression of these genes and length of terminal restriction fragments, but this was not significant (length of terminal restriction fragments vs. TRF1 mRNA; $r=-0.070$, vs. TRF2 mRNA; $r=-0.121$).

TRF gene expression and clinicopathologic factors Relationships of TRF1 mRNA and TRF2 mRNA expression to clinicopathologic factors including menopausal status, clinical stage, histologic type, tumor size, lymph node metastasis and hormone receptor status were examined, disclosing no correlation between expression of TRF1 mRNA and TRF2 mRNA and any clinicopathologic factor except that of TRF1 with tumor size and progesterone receptor status (Table I).

DISCUSSION

In the present study we measured telomerase activity and length of terminal restriction fragments in breast cancers and noncancerous breast tissues. Telomerase activity was detected in 65.8% of breast cancers, but not in any noncancerous sample. This prevalence of telomerase activity is consistent with previous reports.[25–28)] Terminal restriction fragments tended to be longer in noncancerous tissues than in breast cancers, including 13 tumors without detectable telomerase activity. In 7 of 8 patients with sufficient paired breast cancer and noncancerous sample for assays, terminal restriction fragments were shorter in breast cancer tissues than in corresponding noncancerous tissues. However, in 25 breast cancer tissues with detectable telomerase activity, amount of activity did not correlate with length of terminal restriction fragments. Thus, while telomerase is reactivated in breast cancer tissues, which tend to have relatively short telomeres, telomere length is not regulated by telomerase alone.

Telomere length may be regulated by a delicate balance of suppressors (TRFs, TIN2, and Rap1) and accelerators (tankyrase) of telomerase activity, and possibly by other

mechanisms such as recombination.[29, 30] TRF1 is considered to inhibit the action of telomerase at the telomeric region while TRF2 is believed to prevent fusion of chromosome ends; *in vitro*, TRF2 remodels linear telomeric DNA into large duplex loops. TRFs directly bind to the telomeric DNA, but TIN2, Rap1 and tankyrase bind to TRFs instead. In the present study we measured gene expression of TRFs in breast cancers and noncancerous breast tissues. TRF1 mRNA and TRF2 mRNA were both significantly more abundant in noncancerous tissues than in cancers. In addition, significantly more TRF1 mRNA was present in breast cancers without detectable telomerase activity than in cancers with detectable activity. Thus, TRF1 may be particularly important in regulating telomere length of human breast cancers with detectable telomerase activity.

Clinical stage, histology, tumor size, and lymph node metastasis have been identified as prognostic factors in breast cancer patients. In the present study, expression of TRF1 mRNA and TRF2 mRNA generally did not correlate to clinicopathologic factors. An exception was that TRF1 correlated with tumor size and progesterone receptor status. Expression of TRF1 mRNA tended to be decreased in breast cancers in stage II and III ($P<0.055$), and was significantly decreased in tumors 2 cm in diameter or larger ($P<0.018$) and in tumors negative for progesterone receptor ($P<0.043$). No correlation was noted between telomerase activity or length of terminal restriction fragments and clinicopathologic factors (data not shown).

In conclusion, our results suggest that not only reactivation of telomerase activity, but also escape from negative regulation of telomerase activity is needed for a breast cancer to maintain sufficient telomere length for its cells to escape cell death. Genes encoding telomerase inhibitors might be of value in gene therapy against human breast cancer.

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