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DETECTION OF TELOMERASE ACTIVITY IN BREAST CARCINOMA

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Objective: To investigate the significance of telomerase activity in breast carcinoma with its respect to axillary lymph node status. Methods: Telomerase activity was analyzed in 88 breast carcinomas and 16 benign breast lesions, using polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) assay. Results: Telomerase activity was detected in 75 (85%) of 88 breast carcinomas (including three breast carcinomas *in situ* which were all positive for telomerase activity), whereas in benign breast lesions analyzed only 2(12.5%) of 16 cases were positive for telomerase activity. The difference between the two groups was statistically significant ($P<0.001$). Besides, telomerase activity was expressed significantly higher in node-positive breast carcinoma (93%) than in node-negative ones (77%) ($P<0.05$). Conclusion: Our results suggest that telomerase activation plays an important role during breast carcinoma development. It is possible that this enzyme may serve as an early indication of breast carcinoma.

Key words: Telomerase activity, Breast carcinoma, Telomeric repeat amplification protocol (TRAP)

Telomeres are specialized structures at the ends of eukaryotic chromosomes which function in maintaining chromosomal integrity.[1] Human telomeres consist of arrays of tandem repeats of the sequence TTAGGG. Progressive shortening of telomeres with cell division results in chromosomal instability, leading to cellular senescence and death. Telomerase is a ribonucleoprotein which consists of multiple protein subunits and a structural RNA component that contains a template sequence for the telomeric repeat.[2] Recently, telomerase expression has been shown to be closely associated with cellular immortality and cancer. In comparison with benign lesions or normal tissues, telomerase activity was increased in most primary human tumors.[3] In order to investigate the significance of telomerase activity in breast carcinoma with respect to axillary lymph node status, we examined a series of benign breast tissues, *in situ* and invasive breast carcinomas using polymerase chain reaction (PCR)-based telomeric repeat amplification protocol (TRAP) assay.

MATERIALS AND METHODS

Tissue Samples

Eighty-eight breast carcinomas and 16 benign breast lesions were obtained from patients who underwent surgical treatment in our hospital between 1997 and 1998. All samples were obtained within 30 minutes after surgical removal, then frozen and stored in liquid nitrogen. The histological diagnoses are summarized in Table 1.

Preparation of Telomerase Extracts

Telomerase activity was detected with the TRAP

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assay. In brief, frozen tissue specimens were sectioned on a cryostat. Thus, the histological identity of each sample could be checked again by frozen section stained with haematoxylin and eosin. 10 sections, each 10 μm thick, from every specimen were mixed with 400 μl of ice-cold CHAPS (3-[(3-cholamidopropyl) dimethy-ammonio] --1-- propanesulfonate) (Pierce, Rockford, IL) lysis buffer in Eppendorf tubes. After 30 minutes of incubation on ice, the lysate was centrifuged at 14,000g for 30 min at 4°C. Then the supernatant was rapidly frozen and stored at -70°C. Before use, the protein concentration in each lysate was measured by Coomassie brilliant blue assay and adjusted to 5 μg/μl.

Table 1. Results of telomerase activity in breast diseases

Tissue	No. positive/ No. tested	% positive
Benign breast lesion	2/16	12.5
Breast adenosis	0/1	0
Lobular hyperplasia	0/4	0
Adjacent noncancerous Breast tissue	0/4	0
Fibroadenoma	2/6	33
Intraductal papiloma	0/1	0
Breast carcinoma	75/88	85
Invasive ductal carcinoma	62/72	86
Invasive lobular carcinoma	5/7	71
Ductal carcinoma *in situ*	3/3	100
Metaplastic carcinoma	1/1	100
Mucinous carcinoma	1/2	50
Medullary carcinoma	3/3	100

Amplification of Telomeric Repeats

Telomerase activity was assayed by using Oncor TRAP-eze Kit with some modification. Briefly, 1 μl of the extract were incubated in 25 μl of reaction mixture containing TS and TRAP Primer Mix, 20 mM Tris-HCI (PH 8.3), 1.5 mM $MgCI_2$, 63 mM KCI, 0.5% Tween-20, 1 mM EGTA, 50 μM dNTP and 2.5 U of Taq DNA polymerase. After 30 minutes of incubation at 30°C for telomerase-mediated extention of TS primer, the mixture was subjected to 35 cycles of PCR in a Thermal Cycler (Perkin Elmer Cetus) with the following cycle conditions: 94°C 45s, 55°C 45s and 72°C 1 min.

TRAP Product Assay

The PCR products 25 μl were electrophoresed on a 12.5% polyacrylamide non-denaturing gel. The gel was silver-stained, visualized and photographed by IS1000 Imaging Analysis System (Alpha Innotech Cooperation, USA). As a control for the determination of assay specificity, extracts of telomerase-positive tissue specimens were pretreated at 85°C for 10 min to abolish telomerase activity before TRAP. To identify specimens that were non-informative because Taq polymerase inhibitors affected the TRAP assay, an internal control (a 36bp DNA standard) was coamplified with telomerase-elongated products. The internal control was sufficiently short that it did not interfere with the visualization of the telomerase ladder. Tissue samples that did not display telomerase activity were further confirmed by repeating the TRAP assay using 0.05 μg and 0.5 μg of extract protein in the reaction mixtures.

Statistical Analysis

The results were evaluated statistically using χ^2 test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Telomerase Activity in Breast Tissue

Telomerase activity was detected in 75 (85%) of 88 breast carcinomas analyzed (Table 1). The positive samples showed the presence of a six-nucleotide ladder of TRAP products (Figure 1), which were sensitive to heat-pretreatment. However in the control group, 4 adjacent noncancerous breast tissues, 1 breast adenosis, 4 lobular hyperplasia and 1 ductal papilloma were all negative for telomerase activity, whereas 2 of the 6 fibroadenoma showed telomerase activity (Figure 2). The difference of telomerase activity between breast carcinomas and benign breast lesions was statistically significant ($P<0.001$). Telomerase activity of breast carcinomas according to histological type was shown in Table 1.

Telomerase Activity in Breast Carcinoma with Different lymphnode status

In 44 breast carcinoma with metastasis in axillary lymph node, 41 (93%) showed telomerase activity. While in 44 node-negative breast carcinoma, only 34 (77%) cases revealed telomerase activity. The difference was statistically significant ($P<0.05$) Table 2).

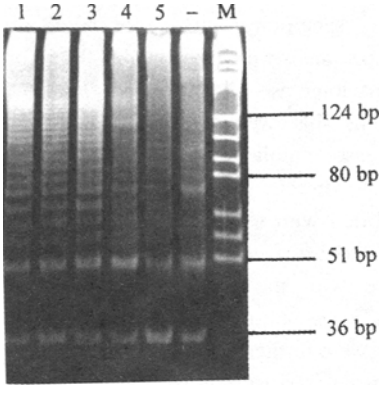

Fig. 1. Results of TRAP assay on breast carcinoma samples.

M: pBR322 HaeIII markers

1-5: breast carcinoma samples, all expressed telomerase activity with the presence of a six-nucleotide ladder of TRAP products. An internal control (36bp) was used to identify false-negative specimens due to inhibitors of Taq polymerase affecting the TRAP assay.

–: Negative control. No obvious ladder was seen.

DISCUSSION

Telomerase activity has been shown to be strongly increased in most human malignancies as compared to benign lesions. Telomerase activity in breast carcinoma was reported by several groups in the world (Table 3). But until now, there remains to be no such report in China. In this work, we have analyzed telomerase activity in benign breast lesions and breast carcinoma. Telomerase activity was detected in 75 (85%) of 88 breast carcinomas analyzed, whereas only in 2 (12.5%) of 16 benign breast lesions ($P<0.001$). What we should emphasize is that the histological diagnosis of each sample was confirmed by examination of a serial cryostat section. This enabled us to omit 3 adjacent noncancerous breast tissue which actually contained carcinoma cells. Our result suggests that telomerase activation plays an important role in breast carcinogenesis.

Table 2. Comparison of telomerase activity with lymph node status

Lymph node metastasis	No. of speci-mens	Telomerase		
		Negative	Positive	% positive
Negative	44	10	34	77
Positive	44	3	41	93
Total	88	13	75	85

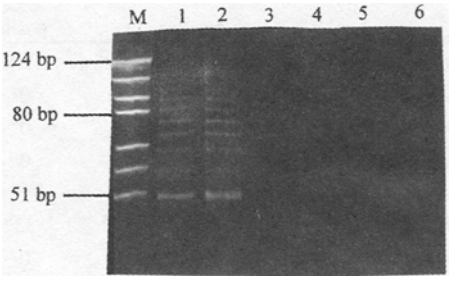

Fig. 2. Results of TRAP assay on benign breast lesions.

M: pBR322 HaeIII markers

1-2: fibroadenomas showed telomerase activity.

3-6: benign breast lesions which were negative for telomerase activity

3-4: lobular hyperplasia 5: breast adenosis

6: breast intraductal papilloma

It is quite interesting that two of the six (33%) fibroadenomas (Fas) had telomerase activity which seems different from other benign breast lesions. Fas are benign biphasic neoplasms composed of benign proliferation of both epithelium and stroma. Although they are usually encapsulated and do not metastasize, they can grow and achieve large size. Hiyama found that 9 (45%) of 20 Fas expressed telomerase. They suggest that the patients who have FA that express telomerase may be at greater risk of subsequent development of breast carcinoma, but this has yet to be confirmed by long-term follow-up of these

patients.[6] We agreed that a transient activation of telomerase may occur in Fas. This activation of telomerase somehow be controlled by estrogens is reversible, so FA may express the enzyme during their growth phase but stop expressing when they reach a stable size or undergo regression and hyalinization. However, additional studies are required to address this issue.

Table 3. Telomerase activity of breast diseases in literature

Author	Breast carcinoma	Adjacent noncancerous breast tissue	Benigh breast lesion	Normal breast tissue
Kim (1994)[4]	79%(19/24)	10%(2/20)		0(0/8)
Sugino (1996)[5]	73%(52/71)		3%(1/32)	0(0/6)
Hiyama (1996)[6]	93%(130/140)	4%(2/55)	2%(9/40)	
Tsao (1997)[7]	76%(37/49)			17%(4/23)
Nawaz (1997)[8]	79%(22/28)		11%(1/11)	0(0/13)

It is thought that ductal carcinoma *in situ*(DCIS) represents an intermediate step in the progression process to invasive breast carcinoma, so it is important to investigate telomerase activity in DCIS lesions. Until now, the reports of telomerase activity in DCIS are quite limited. David reported that neither of two DCIS specimens examined had detectable telomerase activity. In contrast, Marcelo demonstrated that three of three DCIS specimens had telomerase activity which was the same as our results.[6] Tsao also reported 75% of 12 DCIS specimens had telomerase activity.[7] Overall, these results indicate that at least some breast DCIS specimens have telomerase activity. So telomerase activity may be up-regulated during early onset of breast carcinoma.

Hiyama showed an association between telomerase activity and known prognostic factors, such as tumor size, lymph node status and clinical stage.[6] Telomerase activity was detected more frequently in node-positive breast carcinoma than in node-negative ones. Our data supported the findings of Hiyama. We found telomerase activity significantly higher in node-positive than node-negative breast carcinomas. This indicates that telomerase activiny might be a predictive marker for tumor aggressiveness and may distinguish primary tumors with potential for lymphatic spreading from limited local ones. If large series of studies confirm that telomerase could be such a marker, it could become extremely useful in porgnosis. However, several other studies did not confirm these results, and described no correlation between telomerase levels and lymph node status or any other known prognostic indicator. The differences between laboratories may be attributable to minor differences in the conduct of the experimental work. For example the node of protein extraction from the tissues (some using frozen sections, and others using homogenised tissues), the loading of TRAP products onto the gel (some with 8 μl and others with 25μl) and the time of autoradiographic exposure are sometimes different between laboratories.

In summary, our results reveal that telomerase activity can be detected in majority of breast carcinoma, and more frequently in breast carcinoma with positive lymph-node than negative lymph-node. These results suggest that telomerase activation plays an important role during breast carcinoma development. Although the biological significance of telomerase activity in noncancerous breast lesions, such as fibroadenoma is unclear at present, it is possible that this enzyme may serve as an early indication of breast carcinoma.

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