Journal of Zhejiang University SCIENCE B
ISSN 1673-1581
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

A study of the relationship between expression level of TRF1 protein and telomerase activity in human acute leukemia*

SHI Ji-min (施继敏), HUANG He (黄 河)[†‡], CHEN Qiao-fang (陈巧芳), LIN Mao-fang (林茂芳)

(*Department of Hematology, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, China*)

[†]E-mail: hehuang@hzcnc.com

Received Aug. 29, 2005; revision accepted Nov. 23, 2005

Abstract: Objective: To study the expression level of TRF1 (telomeric repeat binding factor 1) protein in human acute leukemia and relationship between expression level of TRF1 protein and telomerase. Methods: A quantitative Western-Blot technique was developed using anti-TRF1[33-277] monoclonal antibody and GST-TRF1 purity protein as a standard to further determine the expression level of TRF1 protein in total proteins extracted from clinical specimens. Results: Bone marrow tissues of 20 acute leukemia patients were studied, 11 healthy donors' bone marrows were taken as a control. The expression level of TRF1 protein was significantly higher ($P<0.01$) in normal bone marrow ((2.217±0.462) μg/μl) than that of acute leukemia patients ((0.754±0.343) μg/μl). But there was no remarkable difference between ALL and ANLL patients ((0.618±0.285) μg/μl vs (0.845±0.359) μg/μl, $P>0.05$). After chemotherapy, TRF1 expression level of patients with complete remission elevated ((0.772±0.307) μg/μl vs (1.683±0.344) μg/μl, $P<0.01$), but lower than that of normal ((2.217±0.462) μg/μl, $P<0.01$). There was no significantly difference after chemotherapy ((0.726±0.411) μg/μl vs (0.895±0.339) μg/μl, $P>0.05$). TRF1 expression level of patients with complete remission is higher than that of patients without complete remission ((1.683±0.344) μg/μl vs (0.895±0.339) μg/μl, $P<0.01$). All samples were determined for telomerase activity. It was confirmed that the activity of telomerase in normal bone marrow was lower than that of acute leukemia patients ((0.125±0.078) μg/μl vs (0.765±0.284) μg/μl, $P<0.01$). There was no significant difference of expression level of TRF1 protein between ALL and ANLL patients ((0.897±0.290) μg/μl vs (0.677±0.268) μg/μl, $P>0.05$). After chemotherapy, telomerase activity of patients with complete remission decreased ((0.393±0.125) μg/μl), but was still higher than that of normal ((0.125±0.078) μg/μl, $P<0.01$). Conclusion: The expression level of TRF1 protein has correlativity to the activity of telomerase ($P<0.001$).

Key words: Acute leukemia (AL), Human telomeric repeat binding factor protein 1 (TRF1), Monoclonal antibody, Expression level of TRF1 protein, Telomerase activity

doi:10.1631/jzus.2006.B0154 **Document code:** A **CLC number:** R733.7

INTRODUCTION

Telomeres are distinctive DNA-protein structures that cap the ends of linear chromosomes. It is very important to keep the chromosomes stabilization. Telomerase activity is closely linked to attainment of cellular immortality, a step in carcinogenesis, while lack of such activity contributes to cellular senescence. Telomerase is activated in more than 85% of malignant tumors (Hiayma *et al*., 1997). Human telomeric repeat binding factor 1 (TRF1) is a telomere associated with proteins and participates in a physiological homeostatic mechanism controlling cellular proliferative potential. TRF1 is involved in negative feedback mechanism that allows telomere shortening by inhibiting the activity of telomerase. Down-regulation of TRF1 expression results in telomere elongation and may be involved in cell immortalization and cancerogenesis. Yamada *et al*.(2000)'s study found expression of TRF1 and TRF2 mRNAs was at higher level in the normal cells than in human malignant hematopoietic cell lines

‡ Corresponding author

* Project (No. 2002CB713704) supported by the National Basic Research Program (973) of China

and/or in patients with acute leukemia (AL). Aragona *et al*.(2000) reported the expression level of TRF1 in malignant tumor was lower than that of normal tissue, inflammation and benign tumor. But expression level of TRF1 proteins in acute leukemia, and the correlativity between TRF1 and activity of telomerase are not available.

In this study based on the monoclonal anti-TRF1$^{33\text{-}277}$ antibody (Huang *et al*., 2002) we will identify the monoclonal antibody TRF1$^{33\text{-}277}$ and its application in acute leukemia, to explore the relationship between the TRF1 and tumor.

MATERIALS AND METHODS

Tissue specimens and reagents

The patients group consisted of 20 patients diagnosed to have acute leukemia (10 men and 10 women; aged 12~70 years, mean of 39.1 years). They were inpatients from the Department of Hematology, the First Affiliated Hospital, Zhejiang University, China. The diagnosis was based on the national standards (8 ALL: 2 ALL-L1, 3 ALL-L2, 3 ALL-L3; 12 ANLL: 7 M2, 1 M3a, 1 M4b, 3 M5b). Eleven healthy volunteers (3 women and 8 men aged 20~35 years, mean of 29.5 years) were recruited as control group.

Lymphocyte separating buffer (Ficoll) was poured into centrifugal tube, and gently mixed with the same volume of specimens (2~3 ml bone marrow with ACD) diluted by PBS buffer. Each mixture was centrifuged at 2000~2500 r/min for 10~15 min, and the supernatant was discarded. The mononuclear cells pellets were washed twice with PBS after transferring to eppendoff tubes, and centrifuged at 1500 r/min for 10 min. After counting, the cellular concentration was adjusted to 1×10^7 or 1×10^6 ml^{-1}, and stored at −80 °C after drying.

Acrylamide, N-N′ bi-methylatic acrylamide, TEMED were purchased from Sangon, China. TRF1$^{33\text{-}277}$ purity protein and anti-TRF1$^{33\text{-}277}$ monoclonal antibody were obtained from our research group (Huang *et al*., 2000; 2002). Telomerase PCR-ELISA kits were from Boehringer Mannheim Company, Germany.

Extraction of cellular protein

The precipitate was resuspended with 5 times volume of precooled protein extracting buffer, and thoroughly shaken to achieve homogeneous mixing. The supernatants were extracted after the mixture was boiled at 100 °C for 10 min and then centrifuged at 12000 g for 30 min. Bovine serum albumin (BSA) was used as quantitative standard. The purity of the protein sample was analyzed by ultra-absorbing method before its storing at –70 °C.

Quantitative Western-Blot

Following Luo *et al*.(1996), 30 μg sample protein was separated on a sodium dodecyl sulfate (SDS)-10% (*w*/*V*) polyacrylamide gel (200 V) before being transferred to a nitrocellulose membrane by electroblotting (100 V, 1 h). After blocking in TBST containing 7% (*w*/*V*) nonfat milk powder, the blots were incubated overnight at 4 °C with the anti-TRF1$^{33\text{-}277}$ antibody, followed by three washes in PBS, incubated again for 1 h then horseradish peroxidase-conjugated sheep anti-mouse antibody and then washed three times with TBST for 2 h, then once again incubated with ECL at moderate temperature for 1 min and processed to sensitization for 20 to 60 s with X-ray film. The signal blot was scanned under Ultroscan XL after developing and fixing. The intensity of signal was calculated on the basis of the area and darkness of the blot. Gemination diluted pure TRF1 protein was taken as the quantitative standard, at concentration of 0.0625 μg/μl, 0.125 μg/μl, 0.25 μg/μl, 0.5 μg/μl, 1.0 μg/μl, 2.0 μg/μl, and 4.0 μg/μl. A standard curve was depicted in every experiment using a group of quantitative standards, and the relative content of TRF1 protein was calculated by this way.

Telomerase activity assay

Extract telomerase and detect its activity according to the manufacturer's instructions in the telomerase PCR-ELISA kit (Boehringer Mannheim Company, Germany).

Data analysis

Data were presented as mean±*SD*. Statistical significance was evaluated with Student's *t*-test.

RESULTS

Set up the standard graph of quantitative Western-Blot

Take the gemination diluted TRF1$^{33\text{-}277}$ purified

protein as the relative quantitative standard, construct the graph with its relevant value of the blot density integral of each purified protein with different dilution multiple. Through this way, the relative content of TRF1 protein of tissue samples was calculated on the basis of its Western-Blot density integral (Fig.1 and Fig.2).

Fig.1 Quantitative Western-Blot assay of GST-TRF1 purity protein
Concentrations of GST-TRF1$^{33\text{-}277}$ fusion protein were 0.0625 μg/μl, 0.125 μg/μl, 0.25 μg/μl, 0.5 μg/μl, 1.0 μg/μl, 2.0 μg/μl, 4.0 μg/μl (from left to right)

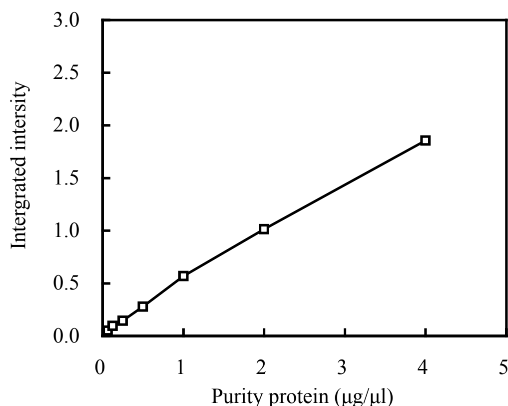

Fig.2 Standard curve of GST-TRF1$^{33\text{-}277}$ purity protein
Concentration of GST-TRF1$^{33\text{-}277}$ purity protein were 0.0625 μg/μl, 0.125 μg/μl, 0.25 μg/μl, 0.5 μg/μl, 1.0 μg/μl, 2.0 μg/μl, 4.0 μg/μl

Quantitative Western-Blot analysis of TRF1 expression of bone marrow tissue

TRF1 was expressed in all AL samples. The level of its expression was variable. Before and after chemotherapy, the expression level of TRF1 was (0.754±0.343) μg/μl and (1.368±0.518) μg/μl, respectively, while the TRF1 expression of samples from healthy donors was (2.217±0.462) μg/μl. The expression level of samples from AL patients was lower than that from normal bone marrow (BM) (albeit after chemotherapy) (P<0.01) (Table 1). Fig.3 shows the result of one group of Western-Bolt patients.

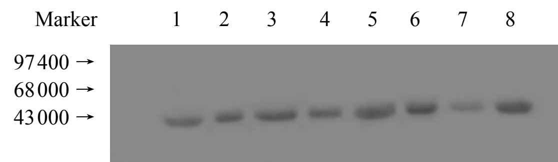

Fig.3 Quantitative Western-Blot assay of the expression level of TRF1 protein in BM tissues of AL patients and normal donors
1, 3, 5, 7 were four specimens of 4 AL patients after chemotherapy; 2, 4, 6, 8 were four specimens of the same 4 AL patients before chemotherapy

Correlativity between TRF1 expression and classifying of AL

In ALL and ANLL patients, the expression level of TRF1 was (0.618±0.285) μg/μl and (0.845±0.359) μg/μl, respectively. The expression level of TRF1 protein between these two types of leukemia was not significantly different (P>0.05).

Correlativity between TRF1 expression and chemotherapy result

After chemotherapy, 12 patients achieved complete remission (CR) while other 8 patients did not. Among patients who achieved CR, the expression level of TRF1 was markedly increased compared with that before chemotherapy ((1.683±0.344) μg/μl vs (0.772±0.295) μg/μl, P<0.01). However, it was still lower than normal BM tissues ((2.217±0.462) μg/μl, P<0.01). In patients without CR, there was no significant difference of the expression level pre- or post-therapy ((0.895±0.339) μg/μl vs (0.726±0.411) μg/μl, P>0.05).

Telomerase activity of AL patients

Before chemotherapy, the telomerase activity of bone marrow in AL patients was (0.765±0.284), which was much higher than normal ones ((0.125±0.078), P<0.05) and patients achieved CR ((0.393±0.125), P<0.01). Before chemotherapy, the telomerase activity in AL patients (0.897±0.290) was higher than that of ANLL patients (0.677±0.268), but without any statistical difference (P>0.05) (Table 2).

Correlativity between TRF1 protein expression and telomerase activity

Analysis of the correlativity between telomerase activity and TRF1 expression level among 11 normal

Table 1 Patients characteristics

Patient No.	Diagnosis	Sex	Age (year)	Response to chemotherapy	Telomerase			TRF1 (μg/μl)		
					1	2	Ratio (2/1)	1	2	Ratio (2/1)
1	ANLL	M	29		0.635	0.538	0.847	0.855	0.947	1.107
2	ALL	M	63		0.786	0.801	1.019	0.650	0.596	0.917
3	ALL	F	20	CR	1.125	0.412	0.366	0.483	1.219	2.524
4	ANLL	M	27		1.323	0.737	0.557	0.139	0.332	2.388
5	ANLL	M	38		0.439	0.587	1.337	1.556	1.422	0.914
6	ALL	F	47	CR	0.624	0.401	0.643	1.055	1.822	1.727
7	ALL	M	18	CR	1.254	0.677	0.540	0.445	1.019	2.290
8	ANLL	M	31	CR	0.639	0.467	0.731	1.112	1.515	1.362
9	ANLL	F	58	CR	0.486	0.203	0.418	0.969	2.070	2.136
10	ANLL	M	37		1.012	0.733	0.724	0.491	0.588	1.198
11	ANLL	M	70	CR	0.615	0.301	0.489	0.962	1.760	1.830
12	ANLL	F	63		0.733	0.601	0.820	0.693	1.041	1.502
13	ANLL	F	50	CR	0.635	0.421	0.663	0.844	1.809	2.143
14	ALL	M	35	CR	0.823	0.401	0.487	0.585	1.973	3.373
15	ALL	F	35	CR	0.667	0.396	0.594	0.804	2.018	2.510
16	ALL	M	12	CR	1.310	0.423	0.323	0.112	1.673	14.938
17	ALL	F	49	CR	0.590	0.416	0.705	0.806	1.319	1.636
18	ANLL	F	31		0.423	0.346	0.818	0.904	1.164	1.288
19	ANLL	F	34	CR	0.397	0.197	0.496	1.092	1.999	1.831
20	ANLL	F	35		0.785	0.463	0.590	0.523	0.904	1.728

M: Male; F: Female; CR: Complete remission; 1: Before chemotherapy; 2: After chemotherapy

Table 2 The results of TRF1 protein expression level and telomerase activity of AL patients and normal bone morrow

	Control	AL (denovo)	AL (after chemotherapy)	ALL	ANLL
n	11	20	20	8	12
TRF1 (μg/μl)	2.217±0.462	0.754±0.343*	1.368±0.518**	0.618±0.285***	0.845±0.359
Telomerase activity	0.125±0.078	0.765±0.284*	1.367±0.518**	0.897±0.290***	0.677±0.268

Normal vs AL (denovo); * $P<0.01$, AL (denovo) vs AL (after chemotherapy); ** $P<0.01$, AL vs ANLL; *** $P>0.05$

bone marrow and 20 AL patients pre- or post-chemotherapy, showed that the expression level of TRF1 protein had negative correlation with telomerase activity, whether in normal BM tissues ($r=-0.891$, $P<0.001$) or in AL patients pre-chemotherapy ($r=-0.881$, $P<0.001$) or in those AL patients post-chemotherapy ($r=-0.820$, $P<0.001$).

DISCUSSION

In human somatic cells, telomeres shorten with successive cell divisions, resulting in progressive genomic instability, altered gene expression, and cell death. As a regulating factor of telomerase, TRF1 have been proposed as candidates for the role of molecules regulating telomerase activity, and have been suggested to play key roles in the maintenance of telomere function. Since TRF1 can specifically bind to the TTAGGG repeat domain and inhibit the telomerase activity by regulating telomere length, it was regarded as a negative regulatory factor (van Steensel and de Large, 1997).

Using the serial diluted TRF purity protein as the relative quantitative standard, we set up a quantitative Western-Blot technique based on anti-TRF1$^{33\text{-}277}$ monoclonal antibody. Each group of samples had a standard curve. Intergrated intersity of samples was converted to the relative content of TRF1 protein by using standard curve. The purity TRF1 protein and samples were assayed with the same method, processed by SDS-PAGE and Western-Blot under the same conditions, so affecting factors were reduced and the results were more credible and comparable.

In our study, the expression levels of TRF1 protein were different in bone morrow of AL patients and normal donors, and found to be much lower in AL patients, in accordance with theory indicating TRF1 has low expression level in tumor tissues as a negative regulator, which result is similar to other result reported in cranial tumor tissues (Aragona *et al*., 2001; La Torre *et al*., 2005). Compared to TRF1 level of AL patients pre-chemotherapy, its expression level was markedly increased when they got CR (P<0.001), but was still lower compared to normal volunteers (P<0.001). In contrast, the expression level of TRF1 protein had no significant difference in patients without CR. These results indicated that the expression level of TRF1 protein correlated with the results of clinical therapy, in that it has the potential use as an index for dynamic monitoring of the chemotherapy effects and illness varieties. TRF1 expression level did not show any significant difference between two types of AL patients (P>0.05), although Ohyashiki *et al*.(2001) reported the expression of TRF1mRNA was higher in ALL patients than ANLL patients. Therefore, it suggested that the TRF1mRNA expression level probably had no consistency with its protein expression.

We also found that the telomerase activity of bone marrow cells in denovo AL patients was higher than normal ones, and that it dropped obviously as the patients achieved CR after chemotherapy but was still higher than normal. Meanwhile, there being no statistically significantly difference of telomerase activity between ALL patients and ANLL patients, suggested that the activation of telomerase was closely correlated to the occurrence or development of AL. And by measuring the activity of telomerase, the chemotherapy effects or illness varieties of AL patients could be monitored dynamically, but still could not distinguish ALL from ANLL.

The relationship between the expression level of TRF1 and telomerase activity in normal volunteers and AL patients revealed that the TRF1 protein expression level had negative correlation to telomerase activity (P<0.001), which indicated that TRF1 may affect cellular biological behaviors by regulating the telomerase activity, although it could regulate the telomere length directly. And this mechanism may probably play an important role in the occurrence and development of tumor.

Our quantitative measurement of TRF1 expression in AL patients and study of the relationship between TRF1 and telomerase activity, provided some useful experiment data for further study of the mechanism of TRF1 involved in the occurrence and development of tumor.

References

Aragona, M., Buda, C.A., Panetta, S., Morelli, M., Giudice, A., Campagna, F.L., Pontoriero, A., Cascinu, S., La Torre, F., 2000. Immunohistochemical telomeric-repeat binding factor-1 expression in gastrointestinal tumors. *Oncol. Rep.*, **7**(5):987-990.

Aragona, M., de Divitiis, O., La Torre, D., Panetta, S., D′Avella, D., Pontoriero, A., Morell, M., La Torre, I., Tomasello, F., 2001. Immunohistochemical TRF1 expression in human primary intracranial tumors. *Anticancer Res.*, **21**(3C):2135-2139.

Hiayma, E., Kodama, T., Shinbara, K., Iwao, T., Itoh, M., Hiyama, K., Shay, J.W., Matsuura, Y., Yokoyama, T., 1997. Telomease activity is detected in pancreatic cancer but not in benign tumors. *Cancer Res.*, **57**(2):326-331.

Huang, H., Chen, Q.F., Lin, M.F., 2000. Cloning and high level expression of telomeric repeat binging factor 1 33-277 in *Escherichia coli*. *Journal of Zhejiang University (Medical Sciences)*, **29**(5):193-194 (in Chinese).

Huang, H., Shi, J.M., Chen, Q.F., Luo, Y., Ding, W., Lou, J., 2002. Preparation and characterization of monoclonal antibody against human telomeric repeat binding factor 1. *Chinese Journal of Hematology*, **23**(12):629-631 (in Chinese).

La Torre, D., de Divitiis, O., Conti, A., Angileri, F.F., Cardali, S., Aguennouz, M., Aragona, M., Panetta, S., D′Avella, D., Vita, G., *et al*., 2005. Expression of telomeric repeat binding factor-1 in astroglial brain tumors. *Neurosurgery*, **56**(4):802-810. [doi:10.1227/01.NEU.0000156468.41461.6F]

Luo, J., Bosy, T.Z., Wang, Y., Yasuda, R.P., Wolfe, B.B., 1996. Ontogeny of NMDA R1 subunit protein expression in five regions of rat brain. *Developmental Brain Research*, **92**(1):10-17. [doi:10.1016/0165-3806(95)00191-3]

Ohyashiki, J.H., Hayashi, S., Yahata, N., Iwama, H., Ando, K., Tauchi, T., Ohyashiki, K., 2001. Impaired telomere regulation mechanism by TRF1 (telomere-binding protein), but not TRF2 expression, in acute leukemia cells. *Int. J. Oncol.*, **18**:593-598.

van Steensel, B., de Large, T., 1997. Control of telomere length by the human telomeric protein TRF1. *Nature*, **385**(6618):740-743. [doi:10.1038/385740a0]

Yamada, K., Yajima, T., Yagihashi, A., Kobayashi, D., Koyanagi, Y., Asanuma, K., Yamada, M., Moriai, R., Kameshima, H., Watanabe, N., 2000. Role of human telomerase reverse transcriptase and telomeric-repeat binding factor proteins 1 and 2 in human hematopoietic cells. *Jpn. J. Cancer Res.*, **91**(12):1278-1284.