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Study on the expression and mutation of human telomeric repeat binding factor (*hTRF1*) in 10 malignant hematopoietic cell lines*

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Abstract: Objective: Detecting the expression and mutation of human telomeric repeat binding factor (*hTRF1*) in 10 malignant hematopoietic cell line cells on the base of determining its genomic structure and its four pseudogenes to clarify if *hTRF1* mutation is one of the factors of the activation of telomerase. Methods: *hTRF1*cDNA sequences were obtained from GenBank, its genome structure and pseudogenes were forecasted by BLAST and other biology information programs and then testified by sequencing. Real-time RT-PCR was used to detect the expression of *hTRF1*mRNA in 10 cell line cells, including myelogenous leukemia cell lines K562, HL-60, U-937, NB4, THP-1, HEL and Dami; lymphoblastic leukemia cell lines 6T-CEM, Jurkat and Raji. Telomerase activities of cells were detected by using telomeric repeat amplification (TRAP)-ELISA protocol. PCR and sequencing were used to detect mutation of each exon of *hTRF1* in 10 cell line cells. Results: *hTRF1* gene, mapped to 8q13, was divided into 10 exons and spans 38.6 kb. Four processed pseudogenes of *hTRF1* located on chromosome 13, 18, 21 and X respectively, was named as Ψ*hTRF1*-13, Ψ*hTRF1*-18, Ψ*hTRF1*-21 and Ψ*hTRF1*-X respectively. All cell line cells showed positive telomerase activity. The expression of *hTRF1* was significantly lower in malignant hematopoietic cell lines cells (0.0338, 0.0108~0.0749) than in normal mononuclear cells (0.0493, 0.0369~0.128) (P=0.004). But no significant mutation was found in all exons of *hTRF1* in 10 cell line cells. Four variants were found in part of intron 1, 2 and 8 of *hTRF1*. Their infection on gene function is unknown and needs further studies. Conclusion: *hTRF1* mutation is probably not one of the main factors for telomerase activation in malignant hematopoietic disease.

Key words: Human telomeric repeat binding factor (*hTRF1*), Expression, Mutation, Genome, Processed pseudogene
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INTRODUCTION

The human telomeric repeat binding factor (*hTRF1*) is a telomeric binding protein (Chong *et al*., 1995). It is a 439 amino acid nuclear protein which recognizes double-stranded repeats of the vertebrate telomeric sequence and is identified as a suppressive regulator of telomerase activity and acts to inhibit elongation of telomeres (van Steensel and de Lange, 1997). It is well known that telomerase activity is up-regulated in malignant tumors such as leukemia, but the mechanism is unclear yet. It was reported that *hTRF1* is weakly-expressed in malignant tumors such as gastric cancers (Aragona *et al*., 2000) and breast cancers (Kishi *et al*., 2001) as well as leukemia, especially acute myelogenous leukemia cells (Ohyashiki *et al*., 2001). As mutation may be the cause of low expression of *hTRF1* and thus have a role in the activation of telomerase, it is very interesting to know whether *hTRF1* gene has some mutations in malignant tumor cells, although mutation study of *hTRF1* has not been reported yet. *hTRF1* gene is located on 8q13 (GenBank accession number AC022893), but

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its genomic structure has not been determined. *hTRF1* was reported to have pseudogenes (Young *et al*., 1997) with exact location and structures still unknown. After mRNA expression of *hTRF1* and telomerase activity were detected in 10 malignant hematopoietic cell lines, then mutation of *hTRF1* gene was analyzed on the basis of determining the genome structure of *hTRF1* gene and its four pseudogenes to investigate if mutation is a causative factor of the low expression of *hTRF1* and high telomerase activity in malignant hematopoietic disease.

MATERIALS AND METHODS

Cells and samples

The 10 malignant hematopoietic cell line cells were myelogenous leukemia cell lines K562, HL-60, U-937, NB4, THP-1, HEL and Dami; lymphoblastic leukemia cell lines 6T-CEM, Jurkat and Raji. NB4 cell line was donated by Professor Chen Sai-juan from Center of Molecular Medicine and Human Genome, Ruijin Hospital. The other 9 cell lines were bought from the Shanghai Institute for Biological Sciences, Chinese Academy of Science. The cell lines were cultured in RPMI1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified atmosphere with 5% CO_2. Fifteen normal samples were extracted from peripheral blood mononuclear cells of bone marrow transplantation donors.

Determination of the genome structure and pseudogenes by PCR

To forecast the genome structure of *hTRF1*, *hTRF1*cDNA (NM_017489) was used to search for htgs database by means of BLASTN 2.2.1 programs provided by NCBI (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov). Exon-intron boundaries were determined according to the classical mRNA splicing principle (GT-AG) with the assistance of the Sequencer 3.0.1 (GeneCodes Corporation) program. Pseudogenes were found in the course of the BLAST. Pseudogenes structures were determined by comparing the sequences of pseudogenes with exons of *hTRF1* using Autoassembler 1.4.1 program (Perkin Elmer). DNA Strider program (Hitachi Software Engineering) was employed to analyze corresponding ORFs (open reading frames).

DNA template for genome structure analysis was extracted from peripheral blood mononuclear cells of bone marrow transplantation donor by using conventional phenol/chloroform method. Primers of exons and pseudogenes were designed by Primer Express 1.0 and synthesized by Shanghai Shenyou Biotechnology Co. Ltd. Primers sequences are shown in Table 1. PCR reactions were carried out in mixtures (25 μl for pseudogenes, 50 μl for exons) containing 10 mmol/L Tris-HCl, pH 9.0, 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs (deoxyribonucleoside triphosphates), 0.1% (*w*/*V*) Triton X-100, 200 μmol/L primers, 50 ng of template DNA, and 1.5 units of high-Fidelity Taq polymerase (Sangon). PCR reactions were subjected to 32~35 cycles of denaturation at 94 °C 30 s, annealing at 55 °C for exon2, exon7, exon9 and exon10, 56 °C for Ψ*hTRF1*-13, Ψ*hTRF1*-21, Ψ*hTRF1*-X, exon1, exon3, exon6 and exon8; 58 °C for exon5; 60 °C for exon4; 65 °C for Ψ*hTRF1*-18 45 s; and polymerization at 72 °C for 1 min, in the GeneAmp PCR system 9600 thermal cycler (Perkin Elmer).

PCR amplicons of exons were gel cut and purified by QIAquick Gel Extraction Kit (QIAGEN) and then sequenced directly. PCR amplicons of pseudogenes were cut from agrose gel, purified by QIAquick Gel Extraction Kit (QIAGEN), and then subcloned into the pGEM-T easy vector (Promega) for sequencing. Nucleotide sequencing was performed on ABI PRISM 377 (Perkin Elmer) in Shenyou Biotech Corporation. Sequencing results were analyzed and compared using the Autoassembler 1.4.1 program.

Quantification of *hTRF1* expression by real-time PCR technique

Harvested cells (1×10^6 cells for one isolation) were washed and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform (Gibco) method and treated with DNase to remove genomic DNA contamination, then stored at –80 °C (Chen *et al*., 2004). cDNA was synthesized from isolated RNA using random hexanucleotides and Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR primer of *hTRF1* was a: 5′ TCTCTCTTTGCCGAGCTTTCC 3′, b: 5′ TGGCAAGCTGTTAGACTGGATAG 3′, probe: 5′ (FAM) CCGCTCCGAGGACTT-

CCGCAGG (TAMRA) 3′, with product length of 106 bp. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as calibrator, whose real-time PCR primer was a: 5′ GGGTGTGAACCATGAGAAGTATGA 3′, b: 5′ CATGAGTCCTTCCACGATACCAA 3′, probe: 5′ (FAM) ACAGCCTCAAGATCATCAGCAATGCCTCCT (TAMRA) 3′, with product length of 127 bp. PCR primers for constructing standard plasmid of both *hTRF1* and GAPDH were designed. *hTRF1*'s primer was 5′ ATGGCAGAAACAGAGAGAAACGAC 3′, b: 5′ TTGCCATTTTCCATACAAACAGC 3′, with product length of 485 bp; GAPDH's primer was a: 5′ TCCCTCCAAAATCAAGTGGG 3′, b: 5′ TTTCTAGACGGCAGGTCAGGTC 3′, with product length of 509 bp. After both PCR products were cloned into pMD18-T vector, the vector was then diluted into four different concentrations to construct the standard curve. Quantitive real-time RT-PCR was performed with 1 μl cDNA, 100 mmol each of dNTP, 3.5 mmol $MgCl_2$, 0.32 mmol of each primer, 0.16 mmol probe and 2.5 U Taq DNA ploymerase (total volume of the reaction was 50 μl). The thermal cycler parameters were: 1 cycle at 94 °C for 2 min followed by 45 cycles at 94 °C, 56 °C and 72 °C for 30 s respectively. The corrected gene expression level was defined as divided by the GAPDH expression level. All samples were tested in triplicate, and the average value was used for quantification.

Telomerase activity measurement

One million cells were re-suspended in 100 μl of lysis buffer (CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid) and incubated for 30 min at 4 °C, and lysates were transferred into fresh Eppendorf tubes and stored at –80 °C until use.

Table 1 Primers of four pseudogenes and 10 exons of *hTRF1*

Fragment name	Primer sequence	Length of amplicons
Ψ*hTRF1*-13	CCATGGGTCCTCAGGTTAATAG	998 bp
	GGGAAACTGGTCTAAAATACTACTGC	
Ψ*hTRF1*-18	TCACCGTTTTAGCCGGGATG	1708 bp
	AGCTGACAAACCTGCCCATG	
Ψ*hTRF1*-21	GTGAAAGGAAGTGGCAACATGTG	1635 bp
	GTCCGGTTGTTGAATTTATAATTCCC	
Ψ*hTRF1*-X	CTGTTCAGCTGCTGATAACATCTG	506 bp
	ATTGAACCCCAAATCATCAGG	
Exon1	GGGATTCGAACAAAGTTCAGC	689 bp
	AGACTGGCGGCAAATTCAAC	
Exon2	TATGCACCAGACACTTGTAGGC	285 bp
	TTACTCAACAAACCACACAGTGG	
Exon3	CAAGAAGAGTACCTAGCACATAGTATACAC	376 bp
	ACCAAAGGTCAAATTTCTGAAGAG	
Exon4	CTCAACAGAGGATTTCAGACTTACCTG	382 bp
	AGGTGTAAGCTACTGCGTCCAGC	
Exon5	TGAGGAGAGGCCAGAAGAAGTC	464 bp
	CACACCTTTCTACAAAACCACAATG	
Exon6	AAAGACCACCTTCGTAACGCTG	505 bp
	TGCACAAGTCATGAAATAACTCTACAG	
Exon7	TGCTTGACTACACCATTAATTATGC	430 bp
	TTGGGCTAACTAGGACCCTGAG	
Exon8	GGACACATCAATTCTCCCCTTC	205 bp
	TTTACCTGTTTTCCATTGCCTTAC	
Exon9	CTTTAATGTCCATAAGCGTTATTCG	318 bp
	TGATCTTTGCACTATACCATACTGC	
Exon10	ACCCTCATTAATTGATGGTCACAG	451 bp
	GAAATGTTTAGAGAAGTCTTCCGTC	

Protein concentration was measured in each extract using the Bio-Rad Protein Assay (Bio-Rad, California, USA). Telomerase activity was assessed using telomerase PCR ELISA kit (Mannheim), according to manufacturer's instructions. Assay was performed on 1 μg of lysates and for 22 PCR cycles. Three duplicates were set up in each sample.

Mutation detection and analysis

PCR product direct sequencing was used to detect mutation of each exon of *hTRF1*, PCR primers are listed in Table 1. Five normal DNA samples were detected and used as normal controls. DNA sequencing results were analyzed and compared using the Autoassembler 1.4.1 program.

Statistical analysis

Mann Whitney U test was used for comparison of TRF1 expression between malignant hematopoietic cell line cells and normal mononuclear cell samples. P values <0.05 were considered significant.

RESULTS

Determination of *hTRF1* genomic structure

From BLAST we found that the genomic DNA of *hTRF1* gene spans 38.6 kb on chromosome 8 (AC022893) and that is divided into 10 exons and 9 introns. The exon-intron boundary was also determined. PCR results and sequence analysis of the 10 exons confirmed what we found (Fig.1).

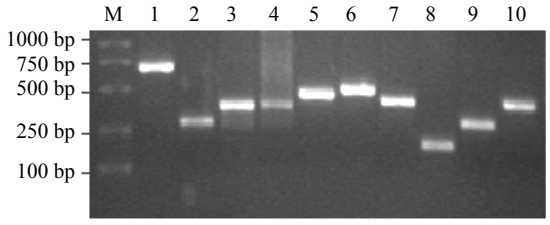


Fig.1 PCR amplicons of the 10 exons of *hTRF1* gene
M: DL-2000 DNA marker (TaKaRa); 1~10: Represent exons respectively

Location and structure of *hTRF1* pseudogenes

We found highly homologous fragments on chromosome 13, 18, 21 and X. They were intronless *hTRF1* genes except for lacking exon7 of *hTRF1*, which was the shortest exon among 10 exons with only 60 bp. All homologous fragments lacked part of exon1 from *hTRF1*. Except for lacking exons, these fragments contained several nucleotide substitutions, insertions and deletions. Their ORFs were different from *hTRF1*; none of the fragments could be translated into a complete and active *hTRF1* protein. In addition, we found a typical polyA sequence at the 3′ flanking region of Ψ*hTRF1*-X, and A-rich sequences at the 3′ flanking regions near the other three pseudogenes. We considered that these homologous fragments contain processed pseudogenes of *hTRF1* (Ψ*hTRF1*-13, Ψ*hTRF1*-18, Ψ*hTRF1*-21 and Ψ*hTRF1*-X). This results is accorded with what had been reported before, although, Ψ*hTRF1*-18 had never been reported. PCR and DNA sequencing results confirmed the existence of the four pseudogenes on chromosome 13, 18, 21 and X (Fig.2 for PCR results).

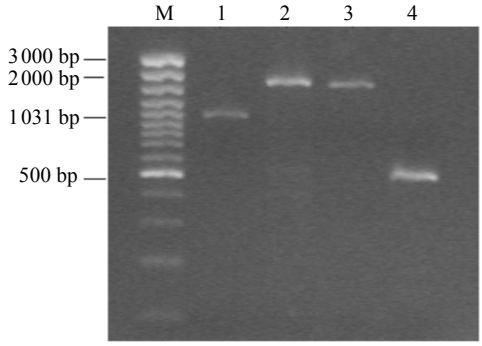


Fig.2 PCR results of the four pseudogenes
M: Molecular sizes (in base pairs) of DNA markers (GeneRuler^{TM} 100 bp DNA Ladder Plus, MBI Fermentas); 1~4: Represent PCR amplicons of Ψ*hTRF1*-13, Ψ*hTRF1*-18, Ψ*hTRF1*-21 and Ψ*hTRF1*-X respectively

Telomerase activity in 10 hematopoietic cell line cells

Telomerase activity of K562 cell was 1.645±0.04, that of HL-60 was 1.756±0.07, of U-937 was 1.503±0.05, of NB4 was 1.298±0.08, of 6T-CEM was 1.648±0.10, of THP-1 was 1.809±0.09, of HEL was 1.796±0.03, of Dami was 1.659±0.03, of Jurkat was 1.705±0.05, and of Raji was 0.643±0.06. In contrast to that of the positive control 1.543 and negative control 0.048, all cell line cells showed positive telomerase activity.

***hTRF1* expression in 10 malignant hematopoietic cell line cells**

*hTRF1*mRNA expression in malignant hemato-

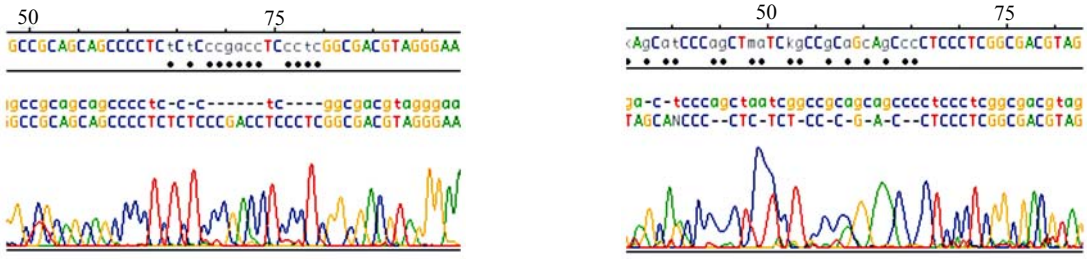
logical cell lines cells (0.0338, 0.0108~0.0749) was significantly lower than that in normal mononuclear cell samples (0.0493, 0.0369~0.128) (P=0.004).

Mutation analysis of *hTRF1* gene in 10 hematopoietic cell line cells

No mutation was found in all exons of *hTRF1* in 10 cell lines, four variants were found in part of intron1, 2 and 8. As for intron1, a deletion of 12 nucleotides located 95 bp downstream of exon1 was found both in normal samples and in malignant hematological cell line cells such as K562, U-937, NB4, HEL, 6T-CEM, THP-1, Jurkat and Raji, but not found in HL-60 and Dami (Fig.3). As for intron2, we found an A to C variant located 11 bp downstream of exon2 in all 10 cell line cells. This variant was also found in 4 of 5 normal samples (Fig.4). As for intron8, two variants were found. One was an A to C variant located 76 bp upstream of exon9, which was found in K562, HL-60, U-937, HEL, THP-1, Jurkat, Dami and 6T-CEM, while Raji cells show a A/C heterozygous mutation while NB4 cells showed no mutation. This variant was not found in 3 of 5 normal samples, the other 2 normal samples showed an A/C heterozygous mutation. Another C to T variant which located 122 bp upstream of exon9 was found in K562, HL-60, U-937, HEL, THP-1, Jurkat, Dami and 6T-CEM cells, while NB4 cells showed a C deleted mutation while Raji cells showed no mutation. This variant was also found in 1 of 5 normal samples (Fig.5).

DISCUSSION

hTRF1 was reported as an inhibitor of telomerase activity and an important telomere length regulator involved in a negative feedback mechanism that stabilizes telomere length: longer telomere would recruit more of *hTRF1*, causing a negative feedback on telomerase and inhibit the enzyme. While short telomere would be lengthened by activated telomerase as they could not bind sufficient *hTRF1* (van Steensel and de Lange, 1997). Low-expression of *hTRF1* would activate telomerase, which could be an



(a) (b)

Fig.3 Variants in intron1 of *hTRF1* (a) Variants in K562, U-937, NB4, HEL, 6T-CEM, THP-1, Jurkat and Raji; (b) Variants in HL-60 and Dami

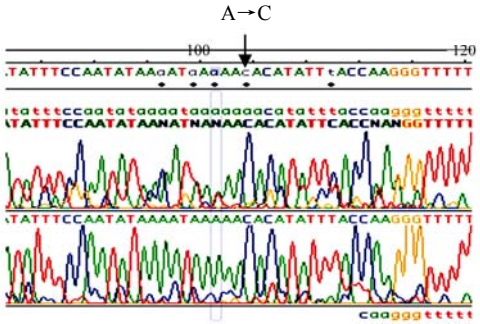


Fig.4 Variant in intron2 of *hTRF1*

An A→C variant located on intron2 of *hTRF1* in K562, HL-60, U-937, NB4, HEL, 6T-CEM, THP-1, Dami, Jurkat, Raji and 1, 3, 4, 5 normal controls, but not in normal control 2

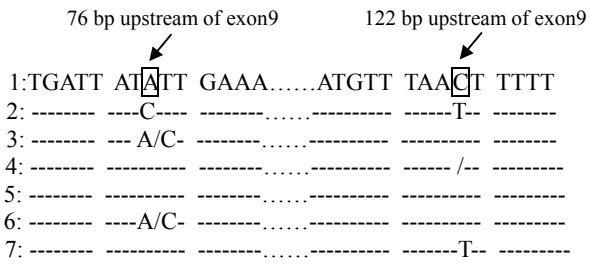


Fig.5 Variants in intron8 of *hTRF1*

1: GenBank sequence; 2: Seqencing results of K562, HL-60, U-937, HEL, THP-1, Jurkat, Dami and 6T-CEM; 3: Sequencing result of Raji; 4: Sequencing result of NB4; 5: Sequencing result of normal control 2 and 5; 6: Sequencing result of normal control 3 and 4; 7: Sequencing result of normal control 1

important step in tumor genesis. Mutation will reduce transcription activity, thus induce low-expression of mRNA. There are some reports on expression of *hTRF1* in leukemia cells (Yamada *et al*., 2002; Ohyashiki *et al*., 2001), but no reports on its mutation in leukemia or other tumors. Its genome structure is undetermined yet.

In this study we determined the genome structure of *hTRF1* at first. *hTRF1* gene locates on 8q13 spans 38.6 kb and divided into 10 exons and 9 introns. We also found four pseudogenes of *hTRF1*: Ψ*hTRF1*-13, Ψ*hTRF1*-18, Ψ*hTRF1*-21 and Ψ*hTRF1*-X. Processed pseudogenes are intronless genomic sequences and always, contain polyA like sequences at their 3′ flanking regions (Vanin, 1985). All four pseudogenes were typical processed pseudogenes. Here, Ψ*hTRF1*-18 is first reported. The determination of pseudogenes helps to remove the disturbance of pseudogenes during the study on *hTRF1* gene.

Yamada *et al*.(2002) studied *hTRF1* expression in 9 malignant hematopoietic cell line cells including K562, HL-60, U-937, RPMI 8226, Ramos, Daudi, BALL-1, MOLT-4, Jurkat and 12 leukemia samples. His results showed that *hTRF1* expression in cell line cells and leukemia samples was significantly less abundant than that in normal control, while telomerase activity was considerably higher. In this study, we detected *hTRF1*mRNA expression by real-time RT-PCR in 10 malignant hematological cell line cells such as K562, HL-60, U-937, NB4, THP-1, HEL, Dami, 6T-CEM, Jurkat and Raji, and detected telomerase activity as well. Real-time RT-PCR is a more sensitive method as it can quantify mRNA expression levels especially when the expression is low. Our result is consistent with reports.

Low mRNA expression may be caused by gene mutation. However, in this study, no mutation was detected in all exons of *hTRF1* gene in 10 malignant hematological cell line cells. So we suppose mutation was probably not the main causative factor for *hTRF1*'s low expression and telomerase activation in malignant hematopoietic diseases. To confirm this conclusion, more samples should be enrolled on *hTRF1*'s mutation detection in the next step of study. In the course of exon mutation analysis, we found four intron variants located on intron1, 2 and 8. As these variants also existed in normal controls, they could be polymorphism sites. Intron mutation or polymorphism located on the gene promoter area or 5′ GT/3′ AG sites may cause aberrant transcription or splicing defect. But none of the four intron variants located on such areas. The significance of these variants is unknown. Here, HL-60 and Dami have different variants with other cell line cells on intron1, while NB4 and Raji have different variants with other cell line cells on intron8. Whether these differences means different gene functions in these cells is unknown and need further studies. Four intron variants are first reported.

The effects leading to *hTRF1* down regulation in malignant hematopoietic cells is unknown. Some factors may act on its promoter and interfere with its transcription, which needs further studies. The causative mechanism of the activation of telomerase is unclear yet. Except for *hTRF1*, many other factors were reported to be involved in telomerase regulation. c-Myc and Sp-1 are concerned with hTERT transcription (Kyo *et al*., 2000). Other telomere binding proteins such as Pinx1 (Zhou and Lu, 2001) and tankyrase (Smith *et al*., 1998) are also regulators of telomerase activity. Telomerase activation may be caused by aberrant behavior of these genes, which will be the next step of investigation.

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