Biol Res 42: 217-222, 2009

Analysis of the nuclear localization signal of TRF1 in non-small cell lung cancer

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ABSTRACT

Several studies revealed a similar down-regulation of telomeric repeat binding factor 1 (TRF1) in tumors. We have previously reported the TRF1 expression levels were down-regulation in non-small cell lung cancer (NSCLC). The regulation of TRF1 localization is proposed to be important for the function and expression. The nuclear localization signal (NLS) and nuclear export signal (NES) are often important clues to localization of protein. The objective of the present study was to investigate the NLS and NES of TRF1 in NSCLC patients. Thirty (30) patients with NSCLCs had undergone radical operations in The First Affiliated Hospital, College of Medicine, Zhejiang University. DNA sequences of NLSs and NESs were amplified by PCR. The PCR products were analyzed by DNA sequencing. There were four NLSs of the TRF1 protein, including two monopartite and two bipartite NLSs. The NLSs sequences were included in 337*KKERRVGTPQSTKKKKESRR*356. The exon 8 and exon 9 of TRF1 DNA **were covered** the NLS sequences. The sequences of predicted NESs were 77*WMLDFLCLSL86* and *174NLIKIQAIAV183*, respectively. The exon 1, exon 3 and exon 4 of TRF1 **were covered** the NES sequences. In NSCLCs, there was no a mutation, deletion, or substitution in NLS and NES of TRF1. We conclude that the NLS and NES sequences in NSCLCs patients did *not* have mutations. Down-expression of TRF1 does not indicate gene mutation of NLS and NES in NSCLCs.

Key terms: non-small cell lung cancer, telomeric repeat binding factor 1, nuclear of localization signal, nuclear of export signal, DNA sequencing.

1. INTRODUCTION

Lung cancer is the most common human malignant tumor. Non-small cell lung carcinoma (NSCLC) accounts for about 80% of all lung cancers. Telomeres are composed of tandem arrays of a short DNA sequence, 5'(GTTAGGG)n3' in vertebrates, and associated proteins [Bianchi et al., 1997]. Telomere maintenance is essential for the continuous growth of tumor cells [Wu et al., 2003]. Telomere-associated proteins are crucial for forming and maintaining the protective telomeric structure. Telomeric repeat binding factor 1 (TRF1), one of the important telomeric binding proteins, plays pivotal roles in telomere protection and maintenance in mammalian cells. In human cells, the TRF1 gene is located on chromosome 8q13 and encodes a ubiquitously expressed protein of 439 amino acids. TRF1 is predominantly located at chromosome ends where they contribute to the protection and maintenance of telomeric DNA [Iwano et al., 2004]. Our previous studies have indicated that TRF1 was often present in reduced quantities in the cancerous tissues of NSCLC compared to its non-cancerous counterparts, suggesting that TRF1 expression is often disrupted during oncogenesis [Hu et al., 2005]. Recently, several studies also revealed a similar down-regulation of TRF1 in tumors

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Grant sponsor: Zhejiang Natural Science Foundation, Grant number: Y206486

Received: July 21, 2009 In Revised form: November 15, 2008 Accepted: April 6, 2009

[Yamada et al., 2002, Yamada et al., 2002, Miyachi et al., 2005, Lin et al., 2006]. These results suggest that down-regulation of TRF1 may be a general phenomenon in cancer tissues. However, the mechanism of TRF1 down expression is not very clear. Previous results showed that TRF1 released from telomeres by tankyrase 1 [Seimiya, 2006]. Chang et al [2003] indicated loss of TRF1 from telomeres results in ubiquitination and degradation of TRF1 by the proteasome and that degradation is required to keep TRF1 off telomeres. Because the down-expression of TRF1 could result from multiple factors, ubiquitination alone is not likely to be the only determinant of TRF1down-expression. When a mutation, deletion, or substitution occurs in the regulatory region of the TRF1, an altered expression is expected. TRF1 expression may also be influenced by nuclear localization signal (NLS) and nuclear export signal (NES), or by other pathways.

Protein import, in general, is specified by NLSs, which are most commonly composed of a short stretch of basic amino acids. The nuclear export signal (NES), a short leucine-rich motif, has been identified as a transport signal that is necessary and sufficient to mediate nuclear export of large proteins. In comparison to protein import, the molecular mechanisms underlying the active nuclear export of proteins are poorly understood. The purpose of *this study* is to *test whether mutations exist* in NLS and NES sequences. If there is mutation in the NLS or NES coding regions of the TRF1 gene, it can lead to an abnormal protein structure and cause an altered function. In this regard, we established the optimal PCR conditions for amplification of the sequences of the TRF1 NLS and NES. Detecting the TRF1 gene of NLS and NES in NSCLC may present a clearer picture of deregulation of the TRF1 in NSCLC.

2. MATERIALS AND METHODS

2.1 Patients and tumor samples

Thirty (30) patients with NSCLCs had undergone curative operations at The First Affiliated Hospital, College of Medicine, Zhejiang University. The diagnosis of lung cancer was obtained by histological examination in all cases. Tissue obtained at the time of surgery was confirmed by pathological examination. Tissue was frozen in liquid nitrogen within 30 min of removal and was then stored at -70° C until use. Paired noncancerous tissue was used as control. Approval from the Committee on Clinical Research of the hospital was obtained.

2.2. Nuclear localization signal and potential of nuclear export signal in TRF1

Human TRF1 has three recognizable domains: an acidic domain at the N-terminus, a conserved TRF-specific domain and a C-terminal domain. hTRF contains two overlapping nucleoplasmin-type nuclear localization signals around position 350, which is rich in aspartic and glutamic acid residues [Chong et al, 1995]. The definition of NLS is based on the analyses by Chong et al, Forwood et al [2002], Smith et al [1999] and the PSORT II for protein sequence analysis. Using these criteria, the NLSs lie within exon 8 and 9. However, the NESs sequence remains poorly defined, and there are currently no high-throughput methods for identifying NESs, so the definition of NES is based on the analyses by the NES Finder 0.2. PSORT II and NES Finder 0.2 were freely available from http: //psort.ims.u-tokyo.ac.jp and http: //research.nki.nl/fornerodlab, respectively. Computer analysis of the primary structure of TRF1 protein revealed the presence of four regions of NLS and two regions of NES basic residues. The TRF1 protein sequence as follow:

MAEDVSSAAPSPRRCADGRDADPTE
EQMAETERNDEEQFECQELLECQVQV
GAPEEEEEEEEDAGLVAEAEAVAAG
WMLDFLCLSLCRAFRDGRSEDFRRTRN
SAEAIIHGLSSLTACQLRTIYICQFLTRIA
AGKTLDAQFENDERITPLESALMIWGSI
EKEHDKLHEEIQ***NLIKIQAIAV***CMENGN
FKEAEEVFERIFGDPNSHMPFKSKLLMII
SQKDTFHSFFQHFSYNHMMEKIKSYVN
YVLSEKSSTFLMKAAAKVVESKRTRTIT
SQDKPSGNDVEMETEANLDTRKSVSDK
QSAVTESSEGTVSLLRSHKNLFLSKLQH

GTQQQDLN***KKERRVGTPQSTKKKKESRR***ATESRIPVSKSQPVTPEKHRARKRQAWLWEEDKNLRSGVRKYGEGNWSKILLHYKFNNRTSVMLKDRWRTMKKLKLISSDSED

NLS basic residues: 337KKER RVGTPQSTKK KKESRR356

NES basic residues: 77WMLD FLCLSL86; 174 NLIKIQAIAV183

Primer designed: NLS sequences correspond to residues 337 to 356 of TRF1. NLSs sequences are contained between the end of exon 8 and 9. Oligonucleotide primers based on NLS sequences were amplified both exon 8 and exon 9 of TRF1.The NES was 77*WMLDFLCLSL*86 and 174*NLIKIQAIAV*183 in TRF1 protein, respectively. The sequences at the exon 1 and also requires sequences in the exon 3 and 4 region.

Primers used in the study were designed with the Primer Premier 5.0 software. The primer sequences were summarized in Table 1.

2.3. TRF1 DNA sequencing analysis of the NLS and NES

Genomics DNA was extracted from carcinoma tissues and paired noncancerous tissues using the DNA Tissue kit (Qiagen,Germany).

PCR amplification: In a total volume of 50μl, 100 ng of genomic DNA extracted from carcinoma tissues or paired noncancerous tissues was amplified in 5μl 10×buffer, 1μl dNTP(10mm/L) 1μl of each primer, 0.2μl Taq polymerase(5U/ul), 35 cycles of denaturation (at 94ºC for 30 s), annealing (40 ^{0}C-60^{0}C for 45s), and extension(at 72ºCfor 50s) were carried out in Techne Touchgene, USA.

DNA purification and DNA sequencing: The product of PCR were purified and sequenced by Invitrogen (Shanghai, China). Sequencing was performed using the same primers as PCR amplification.

Sequence chromatograms were analyzed with a chromas program.

3. RESULTS

DNA sequencing analysis of the TRF1 NLS and NES

The sequence chromatograms are shown in **Fig**. The results show that there is no difference in the NLS and NES of TRF1 between cancer tissues and paired noncancerous tissues. Compared to the sequence of TRF1 in Genbank (NC_000008) and 10 non-neoplastic *patients*, no mutation was found in the NLS and NES of TRF1 in 30 cases.

4. DISCUSSION

TRF1 is a mammalian telomeric protein that binds to the duplex array of TTAGGG repeats at chromosome ends [van Steensel and de Lange 1997]. We have previously reported the TRF1 expression levels were down-regulation in NSCLC. We were interested in why most of the TRF1 protein was often present in reduced quantities in the cancerous tissues of NSCLC compared to its noncancerous counterpart. The

TABLE 1

The primers sequences of NLS and NES

		forward	reverse
NLS	Exon 8	5’ GAGCACCAAAGGAAGTAG 3’	5’ GTAAAGTCAATGGCTGGA 3’
	Exon 9	5’ TAGCTTAGAAAAGGAATT 3’	5’ AAATAAAAGATGGCAAAG 3’
NES			
	Exon 1	5’ GGGATGCCGACCCTACTGA 3’	5’ TGCTGACAACGGGCGAACC3’
	Exon 3	5’ACCTAGCACATAGTATACAC3’	5’AGTATGGCACATTAAGTT 3’
	Exon 4	5’ TTCTCAACAGAGGATTTC 3’	5’ TACTGCGTCCAGCTTATT 3’

abnormal TRF1 expression could result from multiple factors including transcription processing, translation regulation and ubiquitination. TRF1 expression may also be influenced by NLS and NES, or by other pathways. The purpose of *this study* is to *test whether* NLS and NES play roles in regulation of TRF1 expression, so we observed the sequence of NLS and NES.

The cellular localization of protein is closely linked to its NLS **and NES** [Nigg 1997,Gorlich and Kutay 1999]**.** NLS, which can direct protein through the nuclear pore

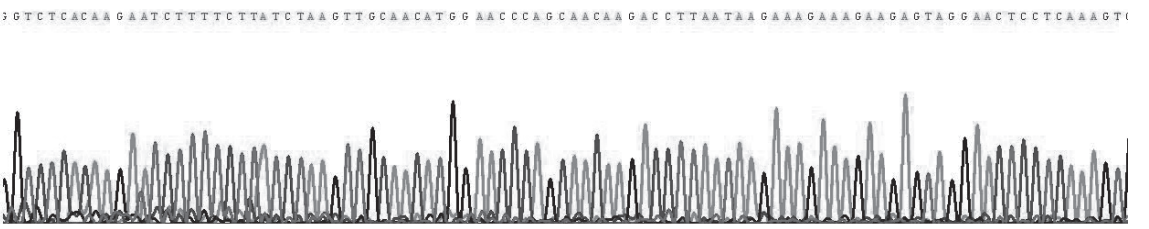

Fig. 1A: Sequencing of exon 8 in sample 15.

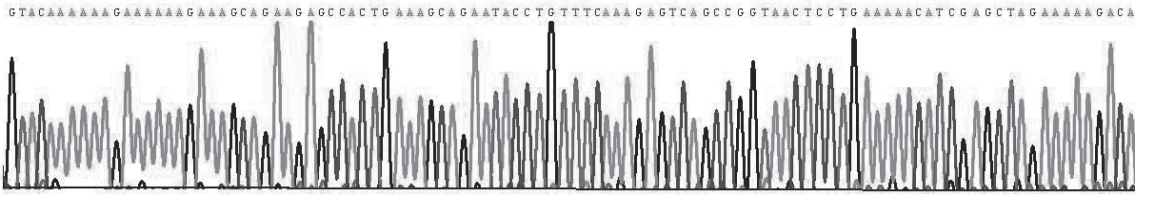

Fig. 1B: Sequencing of exon 9 in sample 15.

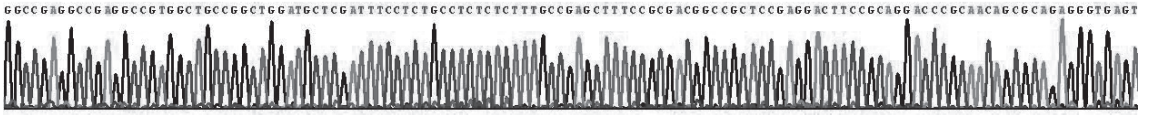

Fig. 2A: Sequencing of exon 1 in sample 15.

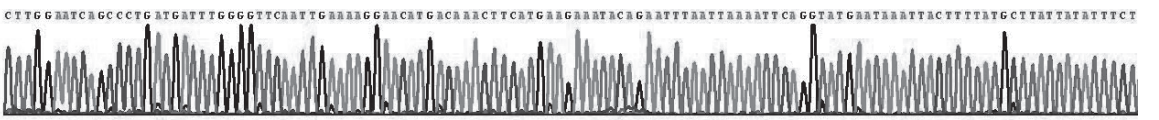

Fig. 2B: Sequencing of exon 3 in sample 15.

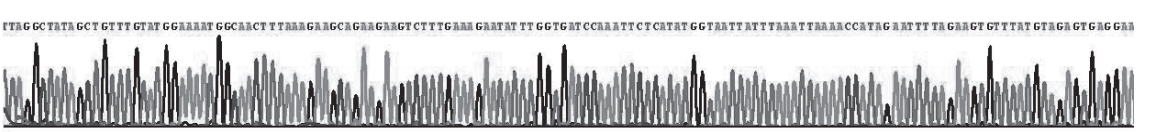

Fig. 2C: Sequencing of exon 4 in sample 15.

complex are characteristically rich in the basic amino acids lysine and arginine. The cellular localization of a protein is indicative of its function. A NLS is a short stretch of amino acids that mediates the transport of nuclear proteins into the nucleus. Another typical NLS, known as bipartite NLS motif contains two interdependent positively charged clusters separated by a mutation tolerant linker region of 10-12 amino acids. Mutation in any basic amino acid cluster of this NLS sequence significantly affects its nuclear localization [Robbins et al.,1991]. Smith et al. [1999] study indicated that, synthesized TRF1, which contains two overlapping bipartite NLSs, could carry the tankyrase protein to telomeres. However, only transfected tankyrase is excluded from the nucleus. The demonstration that co-transfection of TRF1 with tankyrase results in translocation of tankyrase to the nucleus, suggests the TRF1 contains NLSs. In this regard, we used the method of DNA sequencing to examine the mutations of the NLS of TRF1. Because the NLS of the TRF1 included only exon 8 and exon 9, these two exons DNA were sequenced in our study. **However, base mutation was not found in the sequencing of the TRF1 gene of exon 8 or exon 9.** Thus we could not arrive to the conclusion that the mutation of NLS leads to the abnormal localization of TRF1.

Nucleocytoplasmic redistribution of proteins involved in NLS and NES is evidently an important process in many systems [Heerklotz et al., 2001]. NES is a short amino acid sequence of 5-6 hydrophobic residues in a protein that targets it for export from the cell nucleus to the cytoplasm through the nuclear pore complex. It has the opposite effect of NES, which targets a protein located in the cytoplasm for import to the nucleus. The NES is recognized and bound by exportins. A mutation of NES residues prevented protein export. The balance of NLS and NES are decisive for the intracellular localization [**Nguyen** et al., 2002]. Bioinformatic prediction disclosed a putative NLS and a putative NES within human TRF1 protein. The intracellular distribution of TRF1 is evidently caused by the dynamic balance of nuclear import and export. Because the NLS of TRF1 **was not found to be mutant**, in this study, we further examined the NES DNA fragments encompassing exon 1, exon 3 and exon 4, respectively. In NSCLCs, **however, base mutation was not found in the sequencing of the TRF1 gene of exon 1, exon 3 and exon 4**. Thus we could not arrive to the conclusion the mutation of NES leads to the abnormal expression of TRF1.

Savage et al [2005] performed sequence analysis in five Osteosarcoma cell lines and targeted all exons and proximal promoter regions of TRF1, they did not identify mutations in the five Osteosarcoma cell lines studied. Although our study is small in size, it is sufficiently powered to identify common mutations. Our results suggest that there were not mutation, deletion, or substitution occuring in the NLS and NES of TRF1 in NSCLCs. Down-expression of TRF1 does not indicate gene mutation of NLS and NES in NSCLC. However, multiple mechanisms regulate subcellular localization of human protein: NLS, NES and phosphorylation. We further investigated additional studies to clarify this issue.

5. CONCLUSIONS

In conclusion, DNA sequencing results showed that there was no base mutant, deletion, or substitution in NLS and NES of TRF1 in NSCLCs. Down-expression of TRF1 does not indicate gene mutation of NLS and NES in NSCLCs.

Conflict of interest statement

None declared.

ACKNOWLEDGEMENTS

The authors wish to thank all of the patients who so generously contributed their time to this important work. This work was supported by funds from Zhejiang Natural Science Foundation, Grant number Y206486.

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