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Ets2 binding site single nucleotide polymorphism at the *hTERT* gene promoter – effect on telomerase expression and telomere length maintenance in non-small cell lung cancer

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ABSTRACT

The aim of this study was to elucidate the occurrence of DNA sequence changes in the promoter region of *hTERT* gene, and its effect on telomerase expression and telomere length maintenance in non-small cell lung cancer (NSCLC). Between January 2002 and December 2003, 66 NSCLC patients were studied. The expression of *hTERT*, telomerase activity (TA), and *c-Myc* were examined, and the terminal restriction fragment length (TRFL) was measured. A t/n-TRFLR was obtained by dividing the TRFL of the tumour tissue by TRFL of the paired normal tissue. PCR products were sequenced and compared with known *hTERT* gene promoter sequence for a length of 716 bp upstream of the transcription starting code. The changes of any known sequence and/or *c-Myc* expression with their impact on telomerase activity and TRFL maintenance were measured. Positive *hTERT*, TA and *c-Myc* expression was observed in 43 (65.2%), 39 (59.1%) and 59 (89.4%) of the tumour tissue samples, respectively. Except for one patient who had C/C (in normal tissue) homozygotes to T/C (in tumour tissue) heterozygotes point mutation, a novel single nucleotide polymorphism (SNP) −245kb upstream (*Ets2* binding site) of the *hTERT* gene was observed in all normal and tumour tissues, including C/C in 9, T/C in 35, and T/T in 22 of the tumour tissues. The TA of C/C homozygotes was lower than that of T/T homozygotes ($P = 0.0331$), while the t/n-TRFLR of C/C homozygotes was higher than that of T/T homozygotes ($P = 0.0621$). The latter was even more obvious when *c-Myc* were positive ($P = 0.0185$). Our data shows that T/T homozygotes have a lower t/n-TRFLR, but a stronger TA expression, suggesting that the studied *Ets2* binding site is a positive regulator of *hTERT* gene. SNP may interfere with *Ets2* binding and lower TA expression in T/C heterozygotes and C/C homozygotes.

1. Introduction

The human cell telomere reduces in length upon each division at a rate of 50 to 200 bp. This molecular erosion serves as a "mitotic clock" which limits the lifespan of somatic cells.[1] Usually, new telomeric repeats are added to the chromosomal end of the germline cells to maintain their stability and preserve the full genomic information for the next generation.

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Similarly, immortalized cell lines and more than 85% of the cancer cells can prevent the progressive telomere shortening by telomerase activation. Rather than mutations in specific genes, telomere defects also contribute to chromosome instability.[2] Progressive telomere shortening interferes with the formation of telomeric caps which ultimately leads to end-to-end fusion of chromosomes and forms dicentric and multicentric chromosomes. These abnormal chromosomes break during mitosis, causing damage to the genome and the activation of DNA damage checkpoints. Finally, these cells enter a stage of senescence or apoptosis.[3,4] Telomere-induced chromosomal instability can also drive the tumourigenic process by increasing the rate of mutation of oncogenes and tumour suppressor genes.[5]

The proximal promoter of the *hTERT*, especially the proximal 181 bp core promoter, is believed to be responsible for transcriptional activity of the *hTERT* gene.[6] By serial deletion assays of the core promoter, the 5′-region containing E-box, which binds Myc/Max, as well as the 3′-region containing the GC-box, which binds Sp1, have been identified as essential for transactivation. The mutations introduced in the E-box or GC-box significantly decreased transcriptional activity of the promoter. These suggest that c-Myc and Sp1 cooperatively activate the expression of *hTERT* gene.

Our previous study using TRAP assay demonstrated expression of telomerase activity in more than 60–70% of a population of non-small cell lung cancer (NSCLC) patients.[7,8] We also confirmed that it is *hTERT* (not hTR or TP1), which determines the activity of telomerase. Examination of the tumour by Southern blotting also confirms progressive shortening of the TRF (telomeric restriction fragment) length as tumour TNM stage increased.[7] A critical shortening of the tumour TRF length (≦75% of the paired normal tissue) also correlated significantly with survival rates. However, we failed to demonstrate a significant correlation between telomerase expression and the associated genes (c-*Myc*, *TRF1*, and *TRF2*).[7,9] What caused failure in reactivation of the telomerase activity in over 30% of patients is unclear. Mutation or deletion of the binding site for c-Myc/Mad1, Sp1, may contribute to low expression of the telomerase, however, these studies were only conducted using tumour cell lines. We hypothesized that changes in DNA sequence in the promoter region of the *hTERT* gene in resected non-small cell lung cancer tissues may interfere with the activation/depression of *hTERT* gene associate proteins. Hence, we analysed the upstream 800 bp of the *hTERT* promoter DNA sequence. By examining the promoter sequence, in normal and tumour tissues, and telomerase activity in tumour tissues, we analysed the effect of DNA sequence changes on telomerase expression in specific binding sites (c-Myc, SP1, Est2 or GC-box) of the *hTERT* promoter.

2. Materials and methods

2.1. Study population and follow-up

Between January 2002 and December 2003, 66 NSCLC patients (squamous cell carcinoma in 37, adenocarcinoma in 26, and large cell carcinoma in 3) were enrolled in this prospective study. None of the patients received pre-operative chemotherapy or radiotherapy. Pre-operative whole body bone scan and liver sonography were performed on all of the patients to rule out systemic metastasis. The tumour differentiation included well-differentiated carcinoma in 2, moderately differentiated carcinoma in 41, and poorly differentiated carcinoma in 23 cases. Tumour staging was performed according to the AJCC (6^{th} edition) criteria.[10] The p-TNM stages included stage I in 25, stage II in 9, stage III in 29, and stage IV in 3. The demographic data of the patients are listed in Table 1. The study was conducted after human experimentation review by the IRB committee of Taichung Veterans General Hospital, and informed consent was obtained for every examined specimen.

2.2. Tissue sampling and preparation of cell extracts

Tumour samples were acquired from the solid part of the mass, avoiding grossly necrotic areas. The non-tumour bearing paired tissues were acquired from the lobar edge of the resected lung with a distance of at least 5 cm from the gross tumour margin. The resected tissue blocks were preserved in liquid nitrogen for later study. Frozen tissue samples (20 mg) were lysed with 200 μl lysis reagent (Telomerase PCR ELISA Kit) and homogenized by polytron. Samples were then incubated on ice for 30 minutes and the lysate centrifuged at 16000 × g at 4 °C for 20 minutes. The supernatant was transferred to a fresh tube and shock frozen in liquid nitrogen and stored at −70°C. Protein concentration was determined by the Bradford assay (Bio-Rad Protein Assay Kit, Bio-Rad Lab., Hercules, California, USA).

2.3. Genomic DNA extraction

As soon as the specimens were resected from the patients and immediately frozen in liquid nitrogen, they were kept frozen at −70 °C until assay. Frozen tissue (25 mg) was lyzed with 800 μl lysis buffer containing 0.5% (w/v) SDS, 2 mM EDTA (pH 8.0), 0.5 M NaCl, 10 mM $MgCl_2$, 10 mM KCl and 10 mM Tris-HCl (pH 7.6), and digested with proteinase K at 50 μg/ml at 50 °C for at 16 hours. Genomic DNA was extracted from tumour tissues and corresponding normal tissues using Proteinase K digestion and phenol/chloroform extraction. DNA was dissolved in 50 μl H_2O by incubating overnight at 4 °C prior to storage at −70 °C.

2.4. Assay for telomerase activity

Telomerase activity was measured twice in independent experiments using 1–3 μg of total protein. Assays were performed using Telomerase PCR ELISA Kit (Boehringer Mannheim GmbH, Mannheim, Germany) including TRAP assay and detection by ELISA in two steps. In the first step, using TRAP, cell extracts were transferred into a tube with 25 μl reaction mixture, and total volume was adjusted to 50 μl with sterile water. Then those mixtures were incubated at 25 °C for 30 minutes, followed by 94 °C for 10 minutes to inactivate the telomerase. The extended products were amplified by PCR using Taq polymerase, the P1-TS, P2 primers and nucleotides. The PCR conditions were 32 cycles of 94 °C for 30 seconds on a DNA thermocycler (GeneAmp PCR System 9700, Perkin Elmer, Norwalk, CT, USA). In the second

Table 1 – Clinical characteristics of 66 patients with non-small cell lung cancers

	No. of patients
Sex	
Male/Female	55/11
Age	
Range/Mean (years)	30 ~ 84/64.4
Tumour type	
S/A/L	37/26/3
Differentiation	
W/M/P	2/41/23
T-status	
T1/T2/T3/T4	1/40/19/6
N-status	
N0/N1/N2	33/11/22
M-status	
M0/M1	63/3
Stages	
I/II/III/IV	25/9/29/3
hTERT expression	
Positive/Negative	43/23
Telomerase activity	
Positive/Negative	39/27
TRF length (bp)	
Normal/Tumour	5.37 ± 1.63/4.00 ± 1.99
Total	66

S: squamous cell carcinoma; A: adenocarcinoma; L: large cell carcinoma; T: tumour size; N: lymph node; M: Metastasis; W: well; M: Moderately; P: Poorly.

step, using the ELISA method, the amplified products were immobilized onto streptavidin-coated microtiter plates (MTP) via biotin-streptavidin interaction, and then detected by anti-digoxigenin (DIG) antibody conjugated to peroxidase. After addition of the peroxidase substrate (3,3′,5,5′-tetramethyl benzidine), the amount of TRAP products were determined by measurement of their absorbance at 450 nm (with a reference wavelength of 690 nm). Negative control reactions were performed by incubating cell extracts with 1 μg/μl RNase for 20 minutes at 37 °C. The results were interpreted as negative or positive when the OD (optic density) values were ≦0.2 or >0.2 respectively.

Moreover, to confirm the ELISA results, amplified products were systemically run on 15% non-denaturing polyacrylamide gel. After transferring the PCR products onto a positively charged nylon membrane, Southern blotting was performed by the semi-dry electrophoretic blotting instrument (Multiphore II NovaBlot Unit, Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was then incubated with a streptavidin alkaline phosphatase conjugate (1:5000 dilute in blocking solution), and after rinsing, blotted products were visualized by Biotin Luminescence Detection Kit (Boehringer Mannheim, Mannheim, Germany). In addition, all telomerase-negative tumour specimens were re-checked by additional TRAP assay using a 150 bp internal telomerase assay standard to exclude the possibility of Taq DNA polymerase inhibition in the tumour extracts.

2.5. RT-PCR for c-Myc and hTERT genes

Total RNA was isolated from tissue by SV Total RNA Isolation System (Promega Corporation, USA). Complementary DNA (cDNA) was synthesized using 5 μg total RNA with reverse transcriptase (Invitrogen Tech-Linesm, USA) and random primers (Protech Technology Enterprise Co. Ltd.). PCR reaction mixture contained RT-MPCR buffer, 200 mM each of dATP, dCTP, dTTP, and dGTP, 5U Taq DNA polymerase and 1 μl each primer (for Human *c-Myc* and *hTERT*) [RT-MPCR* Kits for Human Telomerase Genes, Cat. No. MP-70140, (Maxim Biotech, Inc., USA)]. The thermal cycles of PCR for human *c-Myc* were performed as follows: 3 cycles at 95 °C for 1 min and 56 °C for 4 min followed by 30 cycles at 94 °C for 1 min and 55 °C for 2.5 min and an extension of 1 cycle at 70 °C for 10 min. The thermal cycles of PCR for human *hTERT* were performed as follows: 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min and an extension of 70 °C for 10 min. PCR products were subjected to electrophoresis through 2.5% agarose gel stained with ethidium bromide.

2.6. Terminal restriction fragment (TRF) length measurement

TRF length measurement was performed using *Telo*TAGGG Telomere Length Assay Kit (Roche, Mannhein, Germany).Genomic DNA (8 μg) was digested with 30U *HinfI*/*Rsa*I at 37 °C for 16 hours. The resulting fragments were separated by electrophoresis on 0.8% agarose gel and transferred to nylon membrane using Southern blotting. After transfer, the transfer DNA was fixed on the membrane by UV-crosslinking (120 mJ). After washing the membrane in 2X SSC, the membrane was first pre-hybridized (DIG Easy Hyb Granules) at 42 °C for 30 minutes and then hybridized with a telomere-specific digoxigenin (DIG)-labeled probe at 42 °C for 16 hours. After washing the membrane in 1X washing buffer, membrane was incubated with 1X blocking buffer at 15–25 °C for 30 minutes and then incubated with anti-DIG-alkaline phosphatase (1:5000 dilute in blocking solution) at 15–25 °C for 30 minutes. Finally, the immobilized telomere probe was visualized by alkaline phosphatase metabolizing CDP-Star, a highly sensitive chemiluminescence substrate. The membrane was then exposed to X-ray film, and the average TRF length was determined by comparing the signals relative to a molecular weight standard (using BIO-PROFIL Bio-1D Software, Version 99, Vilber Lourmat, France), and the mean of three measured TRF lengths deducted by 2.5 kb was used as the presented telomere length.[11,12] Furthermore, the t/n-TRFLR (TRF length ratio) was defined as the ratio between the length of tumour tissue TRF (t-TRF) and their paired normal tissue TRF (n-TRF) from the same patient.

2.7. Primers used in PCR assay of the hTERT promoter

The sequence of the promoter region of the *hTERT* gene was retrieved from Genbank (Accession No. AB016767).[13]

The following sets of over-lapping primers were designed to cover the whole promoter region of the *hTERT* gene as below:

1. Forward-1: 5′-GCAAAGAGAAATGACGGG-3′; −763/+55, 768 bp
2. Forward-2: 5′-ACGTCCGGCATTCGTGGTGGC-3′; −561/+55, 616 bp
3. Forward-3: 5′-CGGGCACAGACGCC-3′; −218/+55, 273 bp
4. Reverse: 5′-GGTAGTGGCTGCGCAG-3′; +55

All of the three mentioned forward PCR primers were used in each tumour and its paired normal tissue for comparison of their end products. Using the amplifying strategy of PCR described below, the length of amplified fragments ranged from 330–800 bp.

2.8. *DNA sequencing analysis*

Genomic DNA, either from tumour tissues or corresponding normal tissues, was amplified with primer pairs covering parts of the promoter region of *hTERT* gene. Each PCR reaction was performed under standard conditions in a 50 μl reaction mixture containing 100 ng of genomic DNA, 5 μl of 10x PCR reaction buffer (500 mM Tris/HCl, KCl, $(NH_4)_2SO_4$, pH 8.3, at 25 °C), 10 μM of each primer, 5 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 10 μl 5xGC-Rich solution, and 0.8 unit of FastStart Taq polymerase (Roche). The reaction mixture was denatured for 4 min at 94 °C and incubated for 35 cycles (denaturing for 30 s at 95 °C, annealing for 30 s at 57 °C, and extending for 50 s at 72 °C). Final extension was continued for 7 min at 72 °C. After amplification and electrophoresis, PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN, USA). Sequencing of the PCR products was carried out using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, USA) according to the manufacturer's recommendations. The automatic DNA sequencer, ABI PRISM™ 3100 genetic analysis system (Applied Biosystems, USA), was used for the sequence analysis. Then the output DNA sequence was compared to the known *hTERT* gene promoter sequence using the data bank provided by BCM Search Launcher-Multiple Sequence Alignments (http://searchlauncher.bcm.tmc.edu/seq-search/nucleic_acid-search.html; Baylor College of

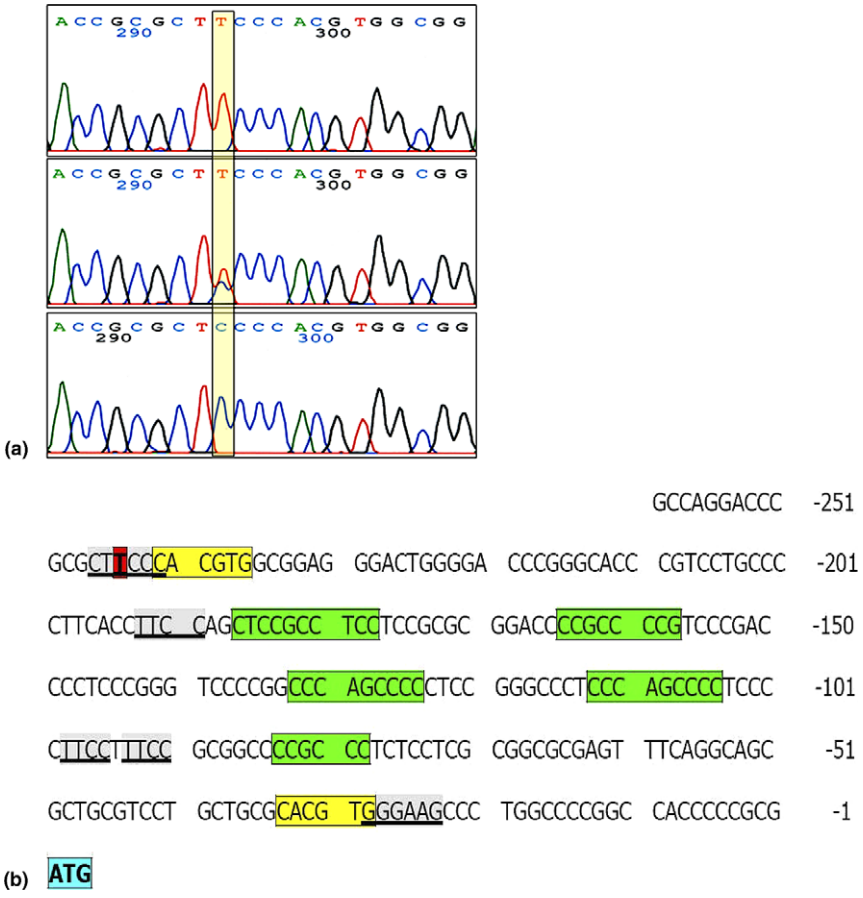

Fig. 1 – a) Three types of SNP (*marked by yellow column*) located at −245 bp upstream of the *hTERT* transcription starting code (ATG) were identified in tumour tissues, including C/C homozygotes in 9 cases, T/C heterozygotes in 35 cases, and T/T homozygotes in 22 cases. b) The sequence of the *hTERT* promoter. Putative protein binding sites for various transcription factors in the first 245 bp are marked. The ATG indicates the first nucleotide 5′ to the start site of transcription. The site of C/T SNP located at −245 bp upstream of the transcription starting code is marked by □, □, E-box, □, SP1 binding site, Ets2 binding site.

Medicine, HGSC), and the NCBI National Center for Biotechnology Information. The known transcription factor binding sites of the *hTERT* gene promoter is shown in Fig. 2.[13,14]

2.9. Statistical analysis

Comparisons between two groups were performed by Pearson's chi-square test and Student's t-test. All probabilities were two-tailed, with P-values less than 0.05 considered statistically significant. All statistical analyses were performed using the SPSS statistical software program package (v11.0 for windows; SPSS, Inc., Chicago, IL).

3. Results

3.1. Patterns of single nucleotide polymorphism in hTERT gene promoter

We identified an single nucleotide polymorphism (SNP) at −245 upstream of the *hTERT* gene transcription start code (see Fig. 1a and b), except for one patient who had C/C homozygotes (in normal tissue) to T/C heterozygotes (in tumour tissue) point mutation, an identical SNP at −245kb upstream (*Ets2* binding site) of the ATG transcription starting code was identified in both of the tumour tissues and their paired normal tissues. The SNP patterns of the tumour tissues included C/C homozygotes in 9 (13.7%), T/C heterozygotes in 35 (53.0%), and T/T homozygotes in 22 (33.3%). The SNP patterns of the normal tissues included C/C homozygotes in 10 (15.2%), T/C heterozygotes in 34 (51.5%), and T/T homozygotes in 22 (33.3%). Except gender, none of the parameters listed in Table 3 associated with SNP at −245 bp site.

3.2. Expression of hTERT gene, c-Myc gene, and telomerase activity

hTERT gene expression and positive telomerase activity was observed in 43 (65.2%), and 39 (59.1%) of the tumour tissues respectively (see Table 1). Fifty-nine (89.4%) patients had positive *c-Myc* expression (see Table 2). The telomerase activity was not affected by the status of *c-Myc* expression (see Table 3). The strength of telomerase activity in tumour tissues as shown by its OD values is listed in Table 4. The T/T pattern had the strongest TA activity as compared with the other patterns (P = 0.089). The P-values of C/C vs, T/C, C/C vs. T/T, and T/C vs. T/T were 0.1343, 0.0331, and 0.2669 respectively. When the expression of *c-Myc* was considered (see Table 5), the T/T and T/C groups had stronger TA activity, compared with the C/C group (P = 0.053). Representative samples showing expression of the TA by TRAP assay, and the associated genes in paired tumour (T) and normal (N) tissues are shown in Fig. 2a and b.

3.3. Terminal restriction fragment length

The mean TRF lengths were 5.37 ± 1.63 kb in normal tissue samples and 4.00 ± 1.99 kb in tumour tissue samples respectively (P <0.0001, T-test). The t/n-TRFLR of individual SNP patterns is listed in Table 5. The T/T pattern had the lowest t/n-TRFLR value as compared with the other patterns (P = 0.107). The P-values of C/C vs. T/C, C/C vs. T/T, and T/C vs. T/T were 0.2577, 0.0621, and 0.3180 respectively. Though the difference did not reach a significant level, a tendency of progressive telomere shortening toward T/T SNP pattern was observed. When the expression of *c-Myc* was considered (see Table 6), the T/T and T/C groups had lower t/n TRFLR

Table 2 – Clinical characteristics of 66 patients with NSCLC and SNP of *hTERT* promoter at −245 bp site

	No. of p'ts	SNP of Tumour at −245 bp			P-values[a]
		C/C	T/C	T/T	
Gender					0.049
Female	11	4	5	2	
Male	55	5	30	20	
Type					0.937
Adenocarcinoma	26	3	15	8	
Squamous cell	37	4	20	13	
Large cell	3	2	0	1	
T factor					0.668
T1 + 2	41	6	23	12	
T3 + 4	25	3	12	10	
N factor					0.207
N0	33	5	14	14	
N1 + 2	33	4	21	8	
M factor					0.764
M0	63	9	33	21	
M1	3	0	2	1	
Stage					0.596
I + II	34	5	16	13	
III + IV	32	4	19	9	
Differentiation					0.666
Well	2	0	1	1	
Moderately	41	5	24	12	
Poorly	23	3	10	10	
Telomerase					0.121
Positive	39	3	20	16	
Negative	27	6	15	6	
hTERT					0.568
Positive	43	7	21	15	
Negative	23	2	14	7	
c-Myc					0.521
Positive	59	9	31	19	
Negative	7	0	4	3	

a Pearson's chi-square test.

Table 3 – Expression of the telomerase activity and changes of t/n-TRFLR according to the status of *c-Myc* expression in non-small cell lung cancers

SNP at −245 bp	TA (OD values)	t/n-TRFLR
c-Myc positive	0.9393 ± 0.1403	0.7205 ± 0.0312
c-Myc negative	1.0706 ± 0.4646	0.8010 ± 0.1072
P-values[a]	0.7944	0.4939

a T-test, two tailed; TA, Telomerase activity; t/n-TRFLR, tumour-to-normal terminal restriction fragment length ratio.

Table 4 – Expression of the telomerase activity and changes of t/nTRFLR according to the SNP at *hTERT* promoter −245 bp upstream of the transcription starting code (ATG) in 66 non-small cell lung cancers

SNP at −245 bp	TA (OD values)	t/n-TRFLR
C/C	0.5303 ± 0.2557	0.8743 ± 0.0269
T/C	0.8931 ± 0.1751	0.8350 ± 0.0266
T/T	1.2219 ± 0.2630	0.7780 ± 0.0387
P-values[a]	0.089	0.107

a Duncan test; TA, Telomerase activity; t/n-TRFLR, tumour-to-normal terminal restriction fragment length ratio. When TA was compared, P-values (t-test) of C/C vs. T/C, C/C vs. T/T, and T/C vs. T/T were 0.1343, 0.0331, and 0.2669, respectively. When t/n-TRFLR was compared, p-values (t-test) of C/C vs. T/C, C/C vs. T/T, and T/C vs. T/T were 0.2577, 0.0621, and 0.3180, respectively.

Table 5 – Expression of the telomerase activity and changes of t/nTRFLR according to the SNP at *hTERT* promoter −245 bp upstream of the transcription starting code (ATG) in 59 c-Myc positive non-small cell lung cancers

SNP at −245 bp	TA (OD values)	t/n-TRFLR
C/C	0.5303 ± 0.2557	0.8132 ± 0.0403
T/C	0.9153 ± 0.2630	0.7455 ± 0.0445
T/T	1.1723 ± 0.1819	0.6358 ± 0.0579
P-values[a]	0.053	0.124

a Duncan test; TA, Telomerase activity; t/n-TRFLR, tumour-to-normal terminal restriction fragment length ratio. When TA was compared, P-values (t-test) of C/C vs., T/C, C/C vs. T/T, and T/C vs. T/T were 0.2366, 0.1114, and 0.4613, respectively. When t/n-TRFLR was compared, p-values (t-test) of C/C vs. T/C, C/C vs. T/T, and T/C vs. T/T were 0.2692, 0.0185, and 0.1417, respectively.

($P = 0.124$). The representative samples showing TRFL in paired tumour (T) and normal (N) tissues are shown in Fig. 3.

4. Discussion

The first Ets (E26 transcription-specific) family member v-ets was identified in 1983 as part of a fusion oncogen in the E26 avian transforming retrovirus.[15] Twenty-seven unique family members have been identified so far with either a transcription activation or suppressive function.[16] The *Ets* domain mediates binding of Ets family members to DNA sequences containing a -GGAA/T- core sequence. More than 200 genes with Ets domain binding sites in their promoters have been reported. With the end products of these target genes, *Ets* transcription factors are involved in various cellular functions, including growth, development, differentiation, adhesion, motility, invasion, and apoptosis.[17,18] Furthermore, evidence implicating cellular transformation and oncogenesis caused by Ets protein has accumulated rapidly.

In addition to telomerase itself, several telomerase-associated proteins and other epigenetic factors are also involved in the regulation of telomerase expression and telomere length maintenance. Regulation of the telomerase activity is a complex process, which involves a number of transcription factors including c-Myc, SP1, Ets family and many other proteins. Among these, Ets proteins interact with Id proteins and c-Myc and cause over/underexpression of the *hTERT* gene. The final influence of these binding proteins may depend upon the different binding sites to which the Ets domain binds. Or, this may be caused by different subgroups of the *Ets* family involved in the binding sites. To determine whether the *hTERT* promoter is the direct target of Ets and how various Ets binding sites on the promoter affect its activity, Xiao designed a 408 bp *hTERT* promoter with Ets binding sites mutation at a different location. They found that both negative and positive Ets regulatory sites exist.[19] Because the Ets inhibitory effect is c-Myc dependent, one can expect that variations in c-Myc levels may further modulate the extent of Ets protein's influence on telomerase expression.[19,20] Though c-Myc, which binds to E-box, plays a major role in *hTERT* activation in many tumours, Ets/Id proteins may be critical in some fine regulation of the telomerase activity due to their effects on *hTERT* promoter. A balance of Ets function together with other transcription factors appears to be crucial for telomerase expression in most cancer cells.

The influence of SNP at the Ets binding site on gene transcription has not been extensively studied. One study identified an SNP of the Ets binding site at −1607 bp in the MMP-1 promoter. With an additional guanine(G), the 2G SNP (5′-GGAT-3′) cells significantly increase the MMP-1 transcriptional power in normal fibroblasts and in melanoma cells.[21] Another report further clarified that this 2G SNP may cooperate with its neighboring AP-1 binding site (at -1602 bp) to enhance transcription in breast cancer cells.[22] There is limited data available concerning the influence of *Ets* binding site mutation or polymorphism on *hTERT* expression and TRF length changes. In this study, we collected the PCR products for sequence analysis, and compared them with the known *hTERT* gene promoter sequence for a length of 716 bp upstream of the transcription starting code in NSCLC. Positive *hTERT* expression and telomerase activity was observed in 43 (65.2%), and 39 (59.1%) of the tumour tissue samples respectively. Discrepancy between *hTERT* gene expression and telomerase activity indicates that *hTERT* gene needs to cooperate with other telomerase associated genes for full function of telomere repair. Although the *hTERT* and TA expression rates were relatively low compared with those reported in other studies, they are consistent with our findings in previous reports.[7,8] Except for one patient who had C/C (in normal tissue) to T/C (in tumour tissue) point mutation, we have identified a SNP at −245kb upstream (*Ets2* biding site) of the ATG transcription starting code in all normal and tumour tissues, including C/C homozygotes in 9, T/C heterozygotes in 35, and T/T homozygotes in 22 of the tumour tissues (see Fig. 1). Except gender, the pattern of SNP is not associated with any clinicopathological factors as shown in Table 3. Males tend to have more frequent T/C and T/T type SNP, however, the clinical significance is not clear. The TA of C/C homozygotes are weaker than that of T/T homozygotes ($P = 0.0331$), while the t/n-TRFLR of C/C homozygotes are higher than that of T/T homozygotes in the current study ($P = 0.0621$). These findings indicate that T/T SNP has a lower t/n-TRFLR, but a higher TA expression, suggesting that SNP at the Ets2 binding

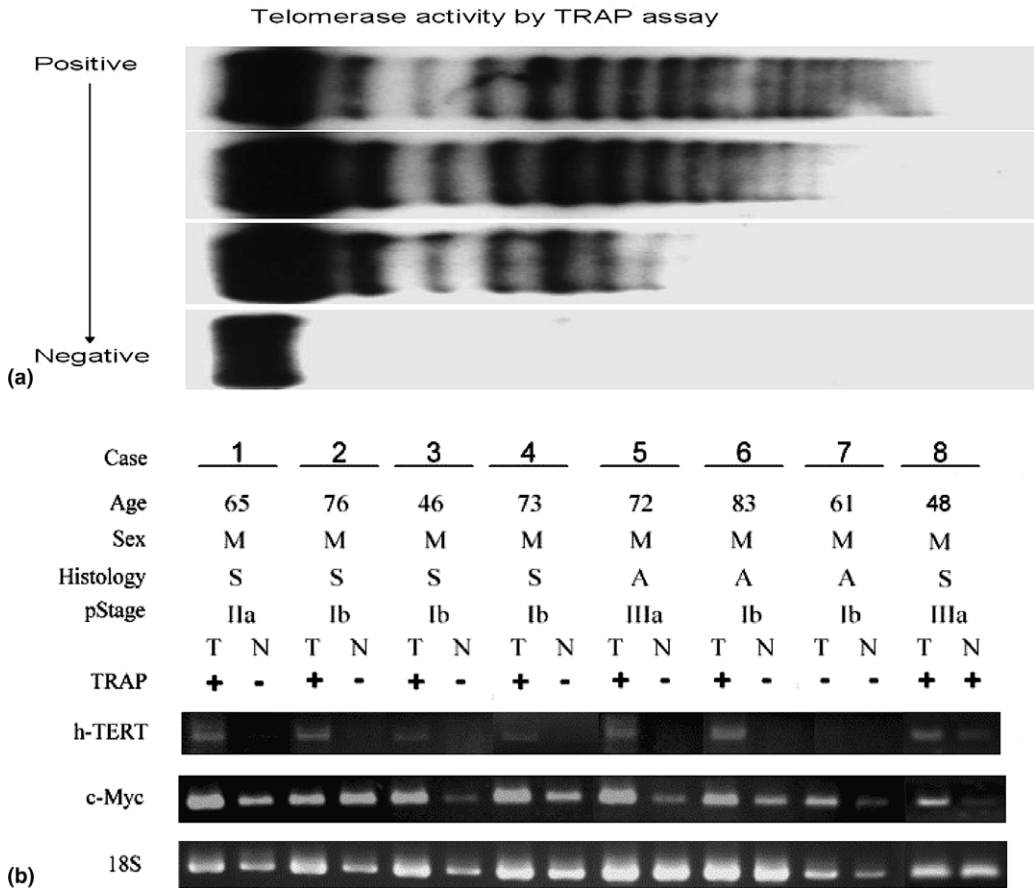

Fig. 2 – a) Representative samples showing different level of telomerase activity (from strong positive to negative) by TRAP assay are shown. b) The RT-PCR products of *hTERT* and *c-Myc* in paired tumour (T) and normal (N) tissues, and the internal controls (18S) are shown according to their telomerase expressions.

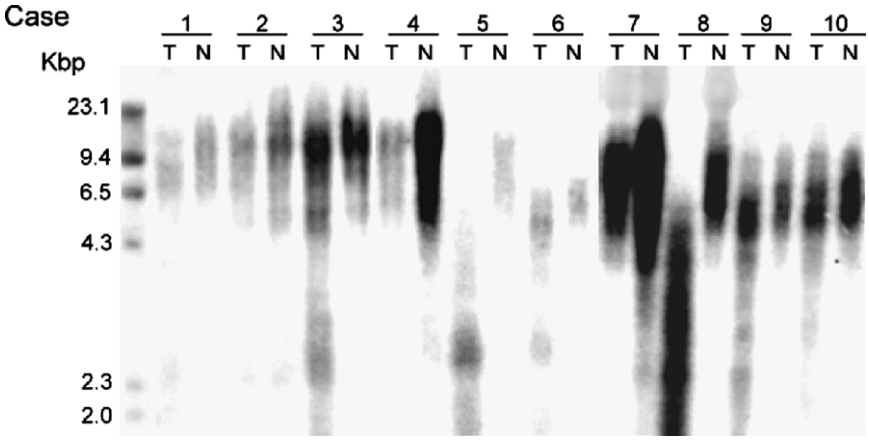

Fig. 3 – Representative samples showing TRFL by telomere length assay kit in paired tumour (T) and normal (N) tissues are shown.

site of the *hTERT* promoter may affect TA expression and TFRL maintenance.

The influence of the Ets binding site SNP on the malignant potential of the tumour is not clear. One report demonstrated that the SNP of the MMP-9 promoter may have a profound impact on the progression and invasion of gastric cancer. However, such a malignant phenotype was not observed in our series, which was similar to another report which investigated SNP of MMP-1 promoter.[23,24]

As shown in Fig. 1b, the Ets2 binding site of the *hTERT* gene promoter may overlap with the c-Myc binding site (E-box). Due to the proximity and/or overlapping of these

two transcription binding sites, it is reasonable to speculate that a competitive or interference mechanism exists, which determines the priority of binding between them. Existing data has demonstrated the interaction between c-Myc protein and Max protein to form a heterodimer to activate the *hTERT* gene transcription by competitive binding with the Mad1 protein at the E-box.[23,25] However, the interaction between the c-Myc and Ets2 binding capability to their individual binding sites has not been elucidated. Because the Ets inhibitory effect is c-Myc dependent, the Ets inhibitory effect on the up-regulation of telomerase activity by c-Myc may be due to its interference with c-Myc binding.[26] If this is the case, then the overall effect of Ets–Myc interplay could be determined by the protein–DNA affinities and/or the protein concentrations. Fifty-nine (89.4%) patients had positive c-Myc expression in this series, but our data failed to demonstrate positive associations between c-Myc expression and telomerase activation or TRFL maintenance. Moreover, in c-Myc positive tumour tissues, the T/T homozygotes had highest TA but lowest t/n-TRFLR values. This suggests that T/T homozygotes which bind to Ets protein (as a positive regulator) may cooperate with c-Myc expression and cause up-regulation of telomerase activity and subsequent TRFL maintenance. However, whether T/T homozygotes have a higher affinity to *Ets* proteins or not is unclear. The current study indicates that Ets proteins probably play as a positive regulator of *hTERT* gene, especially when c-Myc proteins are also expressed. If this is the case, the c-Myc proteins should bind to the proximal E-box and synergically up-regulates *hTERT* expression with Ets proteins. However, there is still possibility that Ets plays as a negative regulator of *hTERT* by competitive binding with c-Myc at the described SNP site. In order to understand the overall interplay of Ets with c-Myc, and to elucidate the inhibitory effect of Ets on the up-regulation of TA by c-Myc (possibly due to its interference with c-Myc binding), it may be useful to determine the protein–DNA affinities using EMSA (Electrophoretic Mobility Shift Analysis) or chromatin IP assay in future studies. Preliminary data in our lab has demonstrated existence of Ets2 proteins that bind to *hTERT* promoter by chromatin IP assay (unpublished data).

In brief, an SNP at −245 upstream of the *hTERT* promoter was identified in NSCLC tumour tissues. Variation in TA expression and t/n TRFLR was observed in different genotypes; the T/T group showed the highest TA and lowest t/n TRFLR. This was even more obvious in c-Myc positive tumour tissues.

Conflict of interest statement

None declared.

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