CHAPTER 2

Materials and methods

2.1 Chemical and reagents

The details of chemicals and reagents are shown in Appendix A.

2.2 Oligonucleotides and probes

All oligonucleotides and fluorescence-tagged oligonucleotides were purchased from Pacific Science and Ward Medic (Thailand). The oligonucleotides were dissolved in TE buffer to have the final concentration of 100 μM (According to the company's label), as a stock DNA solution. The further dilutions were made by ddH_2O to the desired concentration. All oligonucleotides were stored in -20 ^oC prior to use. The DNA sequences for these oligonucleotides are listed in Table 2.1.

2.3 Cell culture

A549 cells, non-small cell lung carcinoma cell line, were grown under 5% CO_2 at 37^oC in 10% FBS DMEM to reach their 80% confluence before being harvested by washing with 1x PBS and trypsinized with 1x trypsin-EDTA. To obtain cell pellet, the cells were washed twice with 1x PBS with centrifugation at 2,000 rpm for 5 min.

Table 2.1 Sequence of oligonucleotides and probe used in this study

Name	Sequence	Product size
Specificity verification of Chr17p-specific primer (17p6)		
17p1F	CCTCAGCCTCTCAACCTGCTT	3,012 bp
17p6	GGCTGAACTATAGCCTCTGC	
GAPDH amplification		
GAPDH forward	TGAAGGTCGGAGTCAACGGATTTGGT	983 bp
GAPDH reverse	CATGTGGGCCATGAGGTCCACCAC	
Internal control synthesis for Chr17p telomere amplification		
17p6-GAPDH forward	GGCTGAACTATAGCCTCTGC TGAAGGTCGGAGTCAACGGATTTGGT	1,023 bp
RP-GAPDH reverse	TAGAGCACAGCCTGTCCGTG CATGTGGGCCATGAGGTCCACCAC	
Chr17p telomere amplification		
RPc3g	TAGAGCACAGCCTGTCCGTG$(CTAACC)_3$GG	-
RP	TAGAGCACAGCCTGTCCGTG	
RP (5' FAM-labeled)	TAGAGCACAGCCTGTCCGTG	
17p6	GGCTGAACTATAGCCTCTGC	
Nested PCR and DIG-labeled Chr17p probe synthesis		
17p1F (5' DIG-labeled)	CCTCAGCCTCTCAACCTCGTT	310 bp
17p1R (5' DIG-labeled)	GAATCCACGGATTGCTTTGTGTAC	
Quantitative (Real-time) PCR		
17pTAS7F	CTGGGAGAGTGTCCATGCTTT	213 bp
17pTAS7R	ATGTGTCTGCTTTCCTGGCAT	
Southern Blotting		
Telo-probe (3' DIG-labeled)	CCCTAACCCTAACCCTAACCCTAA	-
Internal control synthesis for Southern blotting		
17p6-GAPDH forward	GGCTGAACTATAGCCTCTGCTGAAGGTCGGA GTCAACGGATTTGGT	1,021bp
$(C_3TA_2)_3$-GAPDH reverse	$(CCCTAA)_3$CATGTGGGCCATGAGGTCCACCAC	

2.4 Genomic DNA extraction

A549 cells and the leukocytes obtained from 10-mL heparinized blood of human donors were used as the source of DNA. Their DNAs were extracted by using standard protocol with some modifications [62]. For A549 cell pellet, 8 mL of Nuclei Lysis Buffer (NLB) was added to resuspend the pellet. Then, 0.5 mL of 10% Sodium Dodecyl Sulfate was added and mixed vigorously before adding 0.5 mL of Proteinase K solution. The tube was incubated in shaking water bath at 55^{o}C overnight. After that, 5 M NaCl was added at one-fourth of the mixture volume. The tube was kept at 4^{o}C for 20 minutes before centrifugation at 4^{o}C, 3,000 rpm for 25 minutes. The clear supernatant was subsequently transferred to a fresh 50-mL conical tube and added two volumes of ice-cold absolute ethanol. After inverting the tube gently, the DNA was precipitated and suspended in the solution. The DNA was then hooked out, rinsed with 70% ethanol and kept dry at RT for 20 minutes. Finally, the dried DNA was dissolved in Tris-EDTA (TE) buffer, pH 8.0 and measured its concentration by UV-spectrophotometry (GeneQuant™ pro RNA/DNA Calculator Spectrophotometer, GE Healthcare, UK). For leukocytes, buffy coat between plasma and packed red cells were harvested into a fresh 50-mL conical tube after centrifugation the heparinized blood at 1,400xg for 15 min (SC-15AR, TOMY SEIKO CO., LTD., Japan). Forty-five milliliter of Red Cell Lysis Buffer (RCLB) was added and mixed before being centrifuged at 1,500 rpm for 10 min (KOKUSAN, H-103N SERIES). Then, the supernatant was discarded. This step was repeated until the red blood cells were completely lysed and left only WBC pellet. After that, the DNA extraction from the WBC pellet was started at adding NLB and followed with the same steps as that of the A549 pellet. Note that this study was part of the telomere length measurement from blood in Thai population projected, which was approved by the Research Ethics Committee 4, Faculty of Medicine, Chiang Mai University (Study code: BIO-2558-02903).

2.5 Internal control production for chromosome 17p telomere amplification system

One microliter of the 10-fold dilution of 983-bp GAPDH DNA (the PCR product generated from A549's cDNA with GAPDH primers) was used for the 30-µL reaction mixture consisting of 12.5 *p*mol of 17p6-GAPDH forward primer, 12.5 *p*mol of RP-GAPDH reverse primer, 150 µM of dNTPs, 2 unit of *Taq* DNA Polymerase in 1x ViBuffer S Buffer. In the thermocycler (MyGene™ Series Peltler Thermal Cycler Model

MG25+, LongGene®, LongGene Scientific Instruments Co., Ltd., China), the PCR condition was programmed with a cycle of 95ºC for 5 min, followed with 9 cycles of 95ºC for 1 min, 63ºC for 1 min, 72ºC for 1 min and followed with 30 cycles of 95ºC for 1 min, 70ºC for 1 min, 72ºC for 1 min and ended with 72ºC for 7 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by 0.5 µg/mL ethidium bromide. The VC 100 bp plus DNA ladder (Vivantis, USA) was used as reference DNA.

2.6 Internal control production for Southern blot system

The 30-µL reaction mixture [consisting of 1 µL of 1,000-fold dilution of 983-bp GAPDH DNA, 12.5 *p*mol of 17p6-GAPDH forward primer, 12.5 *p*mol of $(CCCTAA)_3$-GAPDH reverse primer, 1 mM of dNTPs, 2 unit of *Taq* DNA Polymerase (Vivantis, USA) in 1x ViBuffer S Buffer] was incubated in the thermocycler. The PCR condition was programmed with a cycle of 95ºC for 5 min, 9 cycles of 95ºC for 1 min, 63ºC for 1 min and 72ºC for 1 min, followed with 30 cycles of 95ºC for 1 min, 67ºC for 1 min and 72ºC for 1 min and ended with 72ºC for 7 min. The PCR product was separated by 1.5% agarose gel electrophoresis and visualized by 0.5 µg/mL ethidium bromide. The VC 100 bp plus DNA ladder (Vivantis, USA) was used as reference DNA.

2.7 DIG-labeled Chr17p probe synthesis

The 20-µL reaction mixture [consisting of 2 µL of 1,000-fold dilution of the 3,072-bp 17p TAS, 10 *p*mol of 17p1F primer, 10 *p*mol of 17p1R primer, 400 µM of dNTPs, 2 unit of *Taq* DNA Polymerase (Vivantis, USA) in 1x ViBuffer S Buffer] was incubated in the thermocycler. The PCR condition was programmed with a cycle of 94ºC for 2 min, followed with 30 cycles of 94ºC for 30s, 63ºC for 30s, 72ºC for 30s, and ended with 72ºC for 7 min. The PCR product was separated by 1.5% agarose gel electrophoresis and visualized by premixed RedSafe™ DNA staining (iNtRON Biotechnology, Korea). The VC 100 bp plus DNA ladder (Vivantis, USA) was used as reference DNA.

2.8 Chr17p telomeric DNA amplification

For FAM fluorescent signal detection by phosphoimager, the 15-µL reaction mixture [containing 250 *f*mol RPc3g, 4.5 *p*mol of 5' FAM-labeled RP, 4.5 *p*mol of 17p6, 5.4 nmol of dNTPs, 1 ng of human leukocyte DNA, and 2 units of non hot-start long-range recombinant Max *Taq* Polymerase (Vivantis) in 1x ViBuffer S buffer] was

incubated in a thermocycler (Mastercycle gradient thermal cycler, BIO-RAD, UK) The 3'-telomeric end extension using RPC3g as DNA template was performed at 3 cycles of 94ºC for 30s, 58ºC for 1 min and 72ºC for 1 min 30s.For the Chr17p telomere amplification, the 25 PCR cycles was conditioned as follow: 94ºC for 15s, 59ºC for 30s and 72ºC for 12 min.

For PCR product detection with chemiluminascence-base Southern blotting, the 15-μL reaction mixture [consisting of 1 ng of genomic DNA, 100 *f*mol of RPc3g, 3 *p*mol of 17p6, 3 *p*mol of RP, 1 mM of dNTPs, 5 mM $MgCl_2$, 0.75 unit of PCRBIO Ultra Polymerase in 1X PCRBIO Ultra Buffer] was incubated in a thermocycler (Masterclycler, Eppendorf). The PCR condition for extending telomeric end with RPC3g DNA adapter was programmed as follows: 95ºC for 5 min, then 55ºC, 53ºC, 51ºC, and 49ºC for 15s each, and 72ºC for 30s. The amplification of Chr17p telomeric DNA was programmed with 25 cycles of 95ºC for 15s, 63.2ºC for 15s, and 72ºC for 10 min. The reaction mixture also contained 1 ng of a 1,023-bp DNA template as an internal positive control. This DNA template can be amplified by the 17p6 and RP primers.

2.9 Genomic DNA digestion

The 20-μL reaction mixture [consisting of 2 μg of genomic DNA, 10 units each of *Hinf*I and *Rsa*I in 1x Tango buffer (ThermoFisher Scientific, USA)] was incubated at 37ºC overnight. The reaction was quenched by 4 μL of 6x loading buffer.

2.10 Southern blotting

In terminal restriction fragment (TRF) length analysis, restriction enzyme digested DNA was separated by 0.8% agarose gel electrophoresis at 50 volts for 3 hr by Mupid®-eXu (Japan) and visualized by premixed 1x RedSafe™ DNA staining while the PCR product from Chr17p telomere amplification was run at 50 volts for 4 hr. Note that the mixture of DNA Molecular Weight Marker III (0.12-21.0 kbp) and VII (0.081-8.57 kbp) (Roche diagnostics, Switzerland) was used as the reference DNA. The vacuum transfer of DNA from the gel onto positively-charged nylon membrane (Immobilon-Ny+ Membrane, MERCK) was carried out with alkaline blotting buffer at 20 mbar for 2 hr. After blotting, the membrane was neutralized with neutralization buffer for 5 min at RT before being submerged in 50 mL of Church buffer (pre-hybridizing solution) at 45ºC for 45 min. The solution was then replaced with 50 mL of hybridization solution containing

400 ng/mL of Digoxigenin (DIG)-labeled telo-probe and incubated at 45ºC for 3 hr (Hybaid Limited Equipment Class 1, UK). The membrane was washed 2 times by 50 mL of stringent buffer I at RT for 5 min a time and then washed 2 times with 50 mL of stringent buffer II at 50ºC for 20 min a time. It was further washed with 50 mL of washing buffer at RT for 5 min before being blocked overnight with 100 mL of 1x blocking solution. The blocked membrane was sensitized at RT for 30 min with 50 mL of 1:10,000 of 0.75 U/mL of alkaline phosphatase-labeled anti-DIG antibody (Anti-Digoxigenin-AP, Fab fragments, Roche Life Science). It was then washed 3 times by 50 mL of washing buffer at RT for 10 min a time to eliminate an excessive anti-DIG-AP. The sensitized membrane was then incubated in 50 mL of 1x detection buffer at RT for 5 min. Then, the membrane was covered with 300 uL of alkaline phosphatase substrate solution (CDP-star™ detection substrate, GE Healthcare Life Sciences) in 4.7 mL 1x detection buffer for 10 min before being exposed with X-ray film. In case of DIG-labeled 17p probe hybridization, every step was the same as those of the TRF, except the hybridizing temperature of 200 ng/mL of DIG-labeled 17p probe (at 50ºC), the temperature for washing with stringent buffer II (55ºC).

2.11 Calculation of average telomere length (mean TRF length)

The average telomere length or mean terminal restriction fragment (TRF) length was calculated according the following formula:

Mean TRF = $\Sigma(OD_i)/ \Sigma(OD_i/L_i)$

Where OD_i is the chemiluminescent signal, L_i is the length of the telomere at position i, i is the DNA molecular weight marker.

2.12 Nested PCR

To verify whether the amplified Chr17p telomeric DNA contained the 17p TAS, 1 μL of the 100-fold dilution of the PCR products was used as DNA template in a 20-μL reaction mixture consisting of 2 units of *Taq* DNA polymerase, 10 *p*mol each of 17p1F and 17p1R primers, 1 μM of dNTPs, 1x ViBuffer S buffer. The PCR condition was programmed with a cycle of 95ºC for 2 min, followed with 35 cycles of 95ºC for 30s, 63ºC for 15s, and 72ºC for 1 min. The PCR products were then separated by 1.5% agarose gel electrophoresis and visualized by premixed RedSafe™ DNA staining. Note that VC 100 bp plus DNA ladder was used as reference DNA.

2.13 Quantitative (real-time) PCR

The 20-μL reaction mixture [consisting of 1 μL of 100-fold PCR product, 10 *p*mol of 17p1F, 10 *p*mol of 17p1R in XPRESS SYBR®GreenER™ qPCR Super Mix (ThermoFisher SCIENTIFIC, USA)] was incubated in the real-time PCR machine (ABI 7500, Applied Biosystems, USA). The temperature program was a cycle of 50°C for 2 min and 95°C for 2 min, 40 cycles of 94°C for 15s, 63°C for 1 min and 72°C for 1 min with signal acquisition. In this experiment, 1 μL of 100-fold dilution of each DNA sample was used to provide the baseline level of amplification and 1 μL of 1,000-fold of the 3,072-bp PCR product from the specificity verification of 17p6 primer experiment was used as internal positive control