CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and reagents

Chemicals and reagents used of this study are described in Appendix A.

2.2 Cells and cell culture conditions

Cell lines used in this study were K562 and U937 (human erythroid leukemia and human monoblastic leukemia cell lines, respectively). These were maintained in RPMI 1640 containing 10 mM HEPES, 1 mM L-glutamine, 100 Units/mL penicillin, 100 μg/mL streptomycin, and supplemented with 10% fetal bovine serum. The cell lines were incubated in humidified 95% air, 5% CO_2 and atmosphere at 37°C.

2.3 Vector construction

The eukaryotic expression vector pcDNA 3.1 containing the cDNA for WT1 [17AA (+) KTS (+)] or WT1 +/+, WT1 [(17AA (+) KTS (-)] or WT +/-, WT1 [(17AA (-) KTS (+)] or WT1 -/+, or WT1 [(17AA (-) KTS (-)] or WT1 -/- and mock control (pcDNA3.1 empty vector) were kindly provided by Professor Dr. Haruo Sugiyama and Associate Professor. Dr. Yusuke Oji, Osaka University Graduate School of Medicine, Osaka, Japan. The eukaryotic expression vector pcDNA 3.1 containing the cDNA GFP (green fluorescence protein) or p95 ErbB2 (676) or ErbB3 or c-Myc-

tagged Lrig1 was kindly provided by Associate Professor Dr. Colleen Sweeney, Cancer Center UC Davis, California, USA.

2.4 Transfection procedure

2.4.1 Stable clone

Plasmid vectors were linearized by restriction enzyme *PvuI* before transfection. U937 cells were resuspended in 10% FBS of RPMI 1640 at a concentration of 5 x 10^4 cells/mL. Five nanograms of plasmid in lipofectin reagent (Qiagen, USA) was added into 2 mL of U937 cell suspension. Cells were incubated at 37^{o}C, 5% CO_2 for 24 h. Then cells were washed with PBS and resuspended in fresh medium, and further incubated at 37^{o}C, 5% CO_2 for 24 h. The cells were cultured in a 96-well plate at the concentration of 1 x 10^3 cells/well (100 µL). After 2 weeks, individual clones that grew in a medium containing G418 (500 µg/mL) (Invitrogen, USA) were expanded for WT1 expression.

2.4.2 Transient transfection

K562 cells (4 x 10^5 cells/mL) were seeded into a 24-well plate and incubated overnight. Then they were transfected with 1 µg of pcDNA3.1 plasmid containing WT1 isoforms or tagged GFP gene. The lipofectamin LTX and PLUS reagents were used for transfection into K562 cells. After 24 h, transfected cells were incubated with or without 15 µM curcumin for 24 h. Then cell lysates were prepared, and protein expression level was detected by Western blot analysis.

2.5 Extraction and isolation of pure curcumin

Pure curcumin was purified from turmeric powder using column chromatography. Pure curcumin extraction and isolation was obtained from the previous preparation (254, 255). Pure curcumin was kindly provided by Associate Professor Dr. Pornngarm Dejkriengkraikul.

Turmeric rhizomes purchased from a local market in Chiang Mai, Thailand were dried and blended to a powder form. The powder was extracted with 95% ethanol for 24 h. The ethanolic extract was filtered through Whatman filter paper No. 2 and ethanol was removed using a rotary evaporator. One kilogram of turmeric powder yielded about fifty grams of laboratory-made crude curcuminoids. Crude curcuminoids were then purified by precipitation with petroleum ether. The precipitate was removed by filtration through Whatman filter paper No. 2 and dried at 60-80°C. HPLC analysis showed that this crude curcuminoid mixture contained 78% curcumin I, 16% curcumin II and 5% curcumin III. Then curcuminoids were further fractionated by silica gel 60 column chromatography, first using $CHCl_3$ and then $CHCl_3$/methanol with increasing polarity, to yield pure fractions of curcumin I, II and III. The fractions were collected and spotted on TLC aluminium sheets coated with silica gel 60 F254. Fractions that showed the same pattern on TLC were pooled and the organic solvent was removed to obtain the powder form. The purity of curcumin I, II and III was in the range of 95-99%, as determined by HPLC analysis. These curcuminoids were used in the experiments described here.

2.6 Proliferation and viability assay

2.6.1 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolim bromide (MTT) assay (256)

MTT assay measures the conversion of tetrazolium 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan in living cells. The reaction is catalyzed by mitochondrial succinate dehydrogenease and requires NADH that must be supplied by the living cells, therefore providing an indication of cell viability. The formazan crystals are then solubilised with dimethyl sulfoxide (DMSO). The solubilised formazan product is spectrophotometically measured using an ELSA microplate reader.

The MTT assay was used to measure the cytotoxicity of the pure curcumin extract on K562 and transfected U937 cell lines. Cells were seeded in flat-bottomed 96-well tissue culture plates. Cell concentration was set to 1 x 10^4 cells/well/100 µL and cultured overnight. Pure curcumin extract of different concentrations was dissolved in 100 µL 10% FBS-RPMI 1640 medium and added in the cells and incubated for 48 h. Then 100 µL of medium was removed and 15 µL of MTT dye (Sigma-Aldrich; USA) was added, followed by a 4-hour incubation. Subsequently, the supernatant from each well was aspirated off, leaving the purple formazan crystals. Optical density was measured by an ELSA microplate reader at 540 nm with a reference wavelength of 630 nm. Percentage of cell survival was calculated by the formula below.

$$\% \text{ Cell survival} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of vehicle control well}} \times 100$$

2.6.2 Trypan blue exclusion assay

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It is a diazo dye. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, trypan blue is not absorbed in a live cell, but it traverses the membrane in a dead cell. Hence, dead cells are seen in a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method.

Cell proliferation was measured by the trypan blue exclusion method. Cells were transfected with pcDNA3.1 or constructed four different WT1 isoforms pcDNA3.1 for 48 h and treated with 25 μM pure curcumin or 0.04% DMSO for 48 h. Then cells were mixed with 0.4% trypan blue dye and counted under microscope. All experiments were performed in triplicate.

2.7 Total RNA extraction and quantitative real time PCR (qRT-PCR)

Real time polymerase chain reaction is also called quantitative real time polymerase chain reaction (Q-PCR/qPCR/qRT-PCR) or kinetic polymerase chain reaction (KPCR). The advent of real time PCR and qRT-PCR has dramatically changed the field of measuring gene expression. Real time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity (257). Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as

cycle threshold (Ct), the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct (258). There are many benefits of using real-time PCR over other methods to quantify gene expression. It can produce quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude (259) and does not require post-amplification manipulation. Real-time PCR assays are 10,000 to 100,000-fold more sensitive than RNase protection assays (260), 1000-fold more sensitive than dot blot hybridization (261), and can even detect a single copy of a specific transcript (262). In addition, real time PCR assays can reliably detect gene expression differences as small as 23% between samples (263) and have lower coefficients of variation (cv; SYBR® Green at 14.2%; TaqMan® at 24%) than end point assays such as band densitometry (44.9%) and probe hybridization (45.1%) (264). Real time PCR can also discriminate between messenger RNAs (mRNAs) with almost identical sequences, requires much less RNA template than other methods of gene expression analysis, and can be relatively high-throughput given the proper equipment.

The qRT-PCR was used in this study to examine WT1 mRNA expression in K562 cells after treatment with pure curcumin or kinase inhibitors. Cells were treated with pure curcumin for 24 h then harvested and isolated using RNeasy® Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The cDNA was synthesized from 5 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA) with random hexamer primers according to the manufacturer's instruction. Quantitative PCR (qPCR) was carried out using TaqMan probe-based chemistry (Operon Biotechnologies GMbH, Germany). The probes for

WT1 and GAPDH (glyceraldehydes-3-phosphate dehydrogenase; internal control) were labelled with FAM and EuroGenetec. The amplification reactions were all performed in triplicate in a BioRad iCycler iQ Real Time PCR instrument. The amplification was performed to activate Taq DNA polymerase at 95°C for 10 min and 40 cycles of sequential denaturation (95°C, 30 sec); annealing (63°C, 60 sec). Data were collected and analyzed with the I cycle™ iQ BioRad program (BioRad, USA). Relative quantitative data based on the $\Delta\Delta C_T$ method (265) were calculated. Normalization: $\Delta C_T = C_T$ (sample) - C_T (GAPDH); $\Delta\Delta CT = \Delta C_T$ (sample 1) - ΔC_T (sample 2). Relative quantification = $2^{-\Delta\Delta CT}$. After RNA extraction, the total RNA extract was analysed for purity and concentration using a spectrophotometer at 260 and 280 nm of absorbance. The purity of RNA was determined by the 260 nm/280 nm absorbance ratio, while RNA concentration was calculated by the formula below.

RNA concentration (µg/mL) = Absorbance 260 nm x 40 µg/mL x Dilution factor

2.8 Protein extraction

2.8.1 Whole protein lysate (crude protein)

The treated cells were washed twice with cold PBS and lysed with cold RIPA buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA) containing protease inhibitor and incubated on ice for 30 min. The cell lysate was centrifuged at 10,000 rpm for 15 min. and the supernatant was collected into new tube and kept in -20°C until use. Whole protein lysate contains membrane, cytosolic, and nuclear proteins.

2.8.2 Nuclear, cytosolic and membrane fractions

Cells were washed twice with ice-cold PBS and then washed again with a hypotonic solution (10 mM 2-hydroxyl piperazinyl ethanesulfonic acid [HEPES]-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT). The cell pellets were resuspended in buffer A (10 mM 2-hydroxyl piperazinyl ethanesulfonic acid [HEPES]-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.1% Nonidet P40) and homogenized using a conical homogenizer and then incubated on ice for 30 min. Cell homogenates were centrifuged at 3,000 rpm for 20 min. The supernatant containing cytosolic and membrane fractions was collected. It was designated as fraction A. The nuclear fraction on the pellets was designated as fraction B.

Fraction A was further centrifuged at 100,000 rpm, 4°C for 1 h. The supernatant was collected and designated as the cytosolic fraction. The pellets were resuspended in RIPA buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA) containing protease inhibitors. It was designated as the membrane fraction.

Fraction B containing cell nuclear material was treated with RIPA buffer and then homogenized on ice. Next, it was further incubated on ice for 30 min. The nuclear homogenates were centrifuged at 10,000 rpm 4°C for 15 min. The supernatant was collected and then it was designated as the nuclear fraction. It was frozen in liquid nitrogen as aliquots and stored at -80°C.

The protein concentration of the nuclear, cytosolic and membrane fractions was measured using Lowry's method (*DC* protein assay kit; BioRad, USA.).

2.8.3 Protein concentration measurement

Protein concentration was measured using the BioRad *DC* Protein Assay. It is a colourimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay, but with the following improvements: the reaction reaches 90% of its maximum colour development within 15 min thereby saving valuable time, and the colour changes not more than 5% in 1 h or 10% in 2 h after the addition of reagents. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to colour development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein (266). Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine.1, 2 Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm (267).

Protein standard curve was constructed by preparing BSA in various concentrations from stock 2 mg/mL BSA, as shown in Table 8. The protein standard solution stock was kept in -20°C. The protein concentration microplate assay was used in this study, five microlitter of the BSA standard protein and protein sample was added into dry microtiter plate and then 25 µL of reagent A (an alkaline copper tartrate solution) was added into each well after that 200 µL of reagent B (a dilute Folin reagent) was added into each well and gently mixed. After 15 min, absorbances were read at 750 nm. The absorbances will be stable for about 1 h.

Table 8 Preparation of stock BSA standard solution

BSA concentration (mg/mL)	Stock BSA (µL)	Deionized distilled water
0	0	1000
0.2	100	900
0.4	200	800
0.8	400	600
1.0	800	200
2.0	1000	0

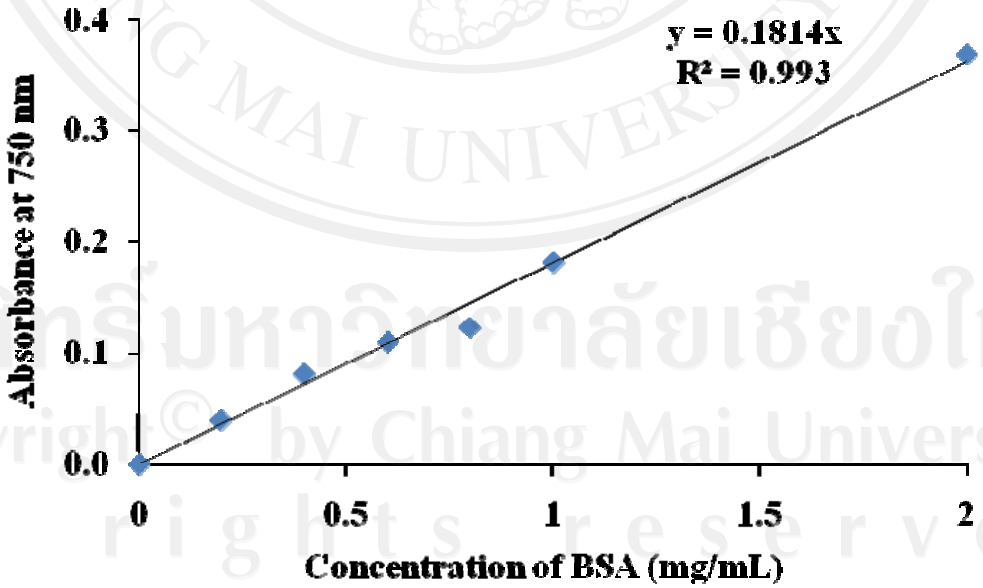

Figure 9 Standard curve of BSA

2.9 Protein detection using SDS-PAGE, Western blot analysis and ECL analysis

2.9.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was applied to investigate the WT1 protein and its molecular mass. The SDS-PAGE procedure separates proteins according to their apparent sizes via the mechanism of the anionic detergent SDS making the surface charge uniformly negative. Applying the nuclear membrane protein extract onto a gel matrix and placing in an electrical field, the negatively charged protein molecules move toward the positively charged electrode at rates dependent on their molecular weight. A small protein, molecule can move through the gel easily and hence migrates faster than a larger molecule. The size of protein can be estimated by comparison of its migration distance with that of a known molecular weight standard protein marker. Acrylamide concentration was set according to the size of a protein to be analysed. Low acrylamide concentrations were used to separate high molecular weight proteins, while high acrylamide concentrations were used for separating low molecular weight proteins. Protein band resolution was improved by using a discontinuous gel system with stacking and separating gel layers.

Electrophoresis was performed as follows; the glass plates were cleaned with detergent, washed with water and ethanol and were allowed to dry. They were aligned and taken to the clamp. The separating gel monomer solution (8% or 12%) was prepared and poured into the glass plates quickly before the acrylamide polymerized. Then the monomer solution was overlaid with 0.1% SDS solution immediately. The gel was allowed to polymerize for 20 min. After that, the overlay solution was completely rinsed off with distilled water. Then the stacking gel monomer solution (4%) was prepared and poured on top of the separating gel. The

comb was then inserted into the staking gel solution and the gel was allowed to polymerize for 15 min. After polymerization was completed, the comb was replaced by pulling it straight up slowly and gently. The wells were completely washed with distilled water. After that, the clamp assembly gel sandwich was released from the casting stand. The gel was placed in the electrophoresis chamber. The electrode buffer was prepared and filled into the reservoir. The pre-running step was performed at 100 volts for 30 min.

The crude protein (70 µg/well) or membrane protein (30 µg/well) of treated or untreated samples, which was prepared by mixing five volumes of the protein sample with one volume of 6X reducing buffer, was loaded into wells under the electrode buffer. Then sample application was carried out using 100 volts by electrophoresis. After that the separated proteins on the gel were transferred to the nitrocellulose membrane by using 30 volts of electricity overnight in transferring buffer. After transblotting, electrophoretically resolved proteins were analyzed by Western blot analysis and ECL detection.

2.9.2 Western blot analysis and ECL detection

After blotting, the membrane was incubated with 5% skimmed milk in 1X PBS for 2 h with shaking at room temperature to block non-specific binding. Then the membrane was incubated with anti-WT1 (C-19) rabbit polyclonal antibody and anti-GAPDH rabbit polyclonal antibody (FL-335) (Santa Cruz, USA) at 1:1,000 dilution in 5% skim milk in 1X PBS with rocking on platform shaker at 4°C overnight. Next day, the membrane was incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Invitrogen, USA) at 1:10,000 dilution in 5% skim milk in 1X PBS for 1 h

with rocking on platform shaker at room temperature. Then the membrane was washed with washing buffer (0.1% PBS-Tween 20) three times to remove excess antibodies. Finally, the protein-antibody complexes were visualized using the SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, USA). This is a two-component substrate that contains a SuperSignal® West Pico Luminol/Enhancer solution and a Stable Peroxidase solution. These two components were mixed together at one-to-one ratio to give sufficient cover to the membrane. The detection reagent was added to the membrane and incubated for 5 min at room temperature. Then the excess buffer was drained and the membrane was removed and put on a piece of plastic wrap. Exportation was detected using Alpha Innotech imaging station with AlphaEase FC Software that was used to capture and quantify images (Cell Biosciences, USA).

2.9.3 Stripping method

The stripping method is a procedure for removing primary and secondary antibodies bound to target proteins on the membrane after Western blotting and ECL detection. The stripping method in this study was used to investigate the GAPDH or β-actin protein expression, a loading control, after WT1 protein was detected.

Stripping methods use conditions that are effective for only low-affinity antibody-antigen interactions or are such harsh conditions that they tend to adversely alter the antigen for subsequent immunoprobing (268). Restore™ Western Blot Stripping Buffer (Thermo Scientific, USA) is a robust but gentle formulation for stripping primary and secondary antibodies from blots to enable several reprobings on the same membrane.

Cold Restore™ Western Blot Stripping Buffer was warmed at room temperature. The membrane was treated with sufficient volume of stripping buffer and then incubated for 15 min at room temperature. After that, the membrane was removed and washed with washing buffer three times, 5 min each. After this step, the membrane was used in Western blot analysis and ECL detection.

2.10 Cytotoxicity of pure curcumin on K562 and stable clone-transfected U937 cells

To investigate the cytotoxicity of pure curcumin on K562 and stable clone-transfected U937 cells, one-hundred microliters of the cell suspension (1 x 10^5 cells/mL) was seeded into a 96-well plate overnight. Next day, various concentrations of 100 µL pure curcumin were added to the cells and incubated for 48 h. The final concentration of pure curcumin ranged from 0-100 µM. Cell viability in each well was determined by MTT assay.

2.11 Effect of pure curcumin on WT1 mRNA levels in K562 and stable clone of transfected U937 cells in a dose dependent manner

Cells were treated with non-toxic concentrations (IC_{20} value) of pure curcumin (5, 10, and 15 µM) for 24 h. DMSO at 0.02% was used as a vehicle control. After incubation, total RNA was extracted and the levels of WT1 mRNA expression were detected by RT-qPCR.

2.12 Effect of pure curcumin on WT1 mRNA levels in K562 and stable clone of transfected U937 cells in a time dependent manner

Cells were treated with an appropriate concentration of pure curcumin (15 μM) for 0, 3, 6, 12, and 24 h. DMSO at 0.02% was used as a vehicle control. After incubation, total RNA was extracted and the levels of WT1 mRNA expression were detected by qRT-PCR.

2.13 Effect of pure curcumin on WT1 protein levels in K562 and stable clone of transfected U937 cells in a dose dependent manner

Cells were treated with non-toxic concentrations (IC_{20} value) of pure curcumin (5, 10, and 15 μM) for 24 h. DMSO at 0.02% was used as a vehicle control. After incubation, crude proteins were extracted and the levels of WT1 protein expression were detected by Western blot analysis as described in section 2.9.2.

2.14 Effect of pure curcumin on WT1 protein expression in K562 and stable clone of transfected U937 cells in a time dependent manner

Cells were treated with an appropriate concentration of pure curcumin (15 μM) for 0, 3, 6, 12, and 24 h. DMSO at 0.02% was used as a vehicle control After incubation, proteins were extracted and the levels of WT1 protein expression were detected by Western blot analysis as described in section 2.9.2.

2.15 Effect of pure curcumin on phosphorylated kinase protein

Cells (2 x 10^5 cells/mL) were treated with 15 μM pure curcumin for 24 h and then harvested and washed twice with ice-cold PBS. After washing, the cells were

lysed with lysis buffer, mixed to resuspend and the extract was rocked gently at 4°C for 30 min. Then the cell lysate was centrifuged at 10,000 rpm for 15 min, and the supernatant was transferred into a clean test tube. Whole protein concentration was measured using Lowry's method (*DC* Protein Assay Kit; BioRad, USA). The membrane containing phosphorylated kinase proteins was treated with blocking buffer for 1 h on a rocking platform. After 1 h, the blocking buffer was removed. Whole protein (500 µg) was added to the antibody-spotted membrane and incubated overnight at 4°C on the rocking platform. Then the membrane was washed with 1X washing buffer three times, for 10 min each. After washing, the membrane was treated with an antibody cocktail (human phospho-kinase antibody) and further incubated for 2 h at room temperature on a rocking platform. Then the membrane was washed three times with 1X washing buffer, for 10 min each. The membrane was treated with Streptavidin-HRP and incubated for 30 min at room temperature on a rocking platform. Then the membrane was washed three times with 1X washing buffer, for 10 min each. The signal was generated by adding an enhanced chemiluminescence reagent (Thermo Scientific, USA) and detected using the Alpha Innotech imaging station with AlphaEase FC Software that was used to capture and quantify images.

2.16 Effect of pure curcumin on activated PKC isoform

Cells were treated with non-toxic doses of pure curcumin (5, 10, and 15 µM) for 24 h. The treated cells were harvested and membrane proteins were extracted as described in section 2.8.2. Membrane proteins (30 µg) were resolved by 12% SDS–PAGE and Western blot analysis. The membranes were probed with a 1:1,000 (v/v)

dilution of primary antibody. The primary antibodies used were anti-PKC rabbit polyclonal antibody isoforms; PKCα, δ, ζ, and PKD/PKCμ (Cell Signaling, USA). The signal was generated by adding an enhanced chemiluminescence reagent (Thermo Scientific, USA) and detected by Alpha Innotech imaging station with AlphaEAse FC Software that was used to capture and quantify images. Caviolin3 was used as an internal control.

2.17 Protein half-life assay

This experiment uses cycloheximide (CHX) to investigate the half-life of WT1 protein. Cycloheximide is a protein biosynthesis inhibitor that inhibits protein synthesis in eukaryotes (but not in prokaryotes) by interfering with the translocation step. It inhibits both chain initiation and chain elongation by acting on the 60S subunit of the eukaryote ribosome, interacting directly with enzyme translocase. It is used as an inhibitor to study cell-free protein biosynthesis in eukaryotes and also used to block ribosome-dependent *in vivo* polypeptide synthesis. It induces apoptosis in a variety of cells, but can also delay or inhibit apoptosis by other agents.

Cells were incubated with 50 μg/mL cycloheximide in the presence or absence of 15 μM curcumin for 0, 3, 6, 12, and 24 h. After that, the cells were lysed in lysis buffer. Crude protein lysate was resolved by 12% SDS–PAGE. Membranes were probed with a 1:1,000 (v/v) dilution of the primary antibody. Primary antibody used in this experiment was anti-WT1 (C-19) rabbit polyclonal antibody. The signal was generated by adding an enhanced chemiluminescence reagent (Thermo Scientific, USA). GAPDH was used as an internal control.

2.18 Proteasome assay

This experiment was performed to confirm WT1 protein half-life by using three proteasome inhibitors, MG132, (-)-epigallocatechin gallate (EGCG), and lactacystin. The MG132 is a cell-permeable, potent and reversible proteasome inhibitor. It reduces degradation of ubiquitin-conjugated proteins in mammalian cells and permeable strains of yeast by the 26S complex without affecting its ATPase or isopeptidase activities. The EGCG is a polyphenol flavonoid that displays antitumor and antioxidant properties. It inhibits telomerase and DNA methyltransferase (DNMT), and blocks the activation of EGF receptors and HER-2 receptors. Moreover, it also potently and specifically inhibits chymotrypsin-like activity of the proteasome *in vitro* and *in vivo*. Lactacystin is a potent and selective inhibitor of the 20S proteasome. It binds irreversibly to the active site N-terminal threonine residue of the catalytic β-subunit of the 20S proteasome, thereby inhibiting its chymotrypsin and trypsin-like activities.

Cells were incubated with or without 5 μM MG132 or 10 μM (-)-epigallocatechin gallate (EGCG) or 10 μM lactacystin in the presence or absence of 15 μM pure curcumin for 12 h. Treated cells were lysed in lysis buffer. Crude protein was loaded into 12% SDS-PAGE. Membranes were probed with a 1:1,000 (v/v) dilution of primary antibody. The primary antibody used in this experiment was anti-WT1 (C-19) rabbit polyclonal antibody. The signal was generated by an enhanced chemiluminescence reagent (Thermo Scientific, USA). GAPDH was used as an internal control.

2.19 mRNA stability assay

Cells were pretreated with 4 μM actinomycin D (Act D; transcription process inhibitor) for 1 h. After that, cells were treated with actinomycin D in the presence or absence of 15 μM curcumin for 0, 6, and 12 h. Total RNA was extracted from the cells using RNeasy® Mini Kit. cDNA was synthesized from 5 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit. The cDNA levels of WT1 mRNA were measured using TaqMan probe for human WT1 on BioRad iQcycle. *GAPDH* or *β actin* gene was used as internal control.

2.20 Chromatin immunoprecipitation (ChIP) assay

The concept of Chromatin immunoprecipitation or ChIP is an experimental form of immunoprecipitation used to investigate the interaction between protein and DNA in the cell. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factor on its promoters or other DNA binding sites, and possibly defining cistromes. Also, ChIP aims to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone modifiers (269). The method, briefly: protein with chromatin in a cell lysate is temporarily bonded, the DNA-protein complexes (chromatin-protein) are then sheared and DNA fragments associated with the proteins of interest are selectively immunoprecipitated, then the associated DNA fragments are purified and sequences are determined (Figure 10). These DNA sequences are supposed to be associated with the protein of interest *in vivo*

The ChIP assay was used to examine the effect of pure curcumin on binding of WT1 transcription fraction and WT1 promoter in K562 cells. Cells were treated with

15 μM pure curcumin for 24 h, followed by 37% formaldehyde (final concentration is 0.37%) for 10 min in room temperature to crosslink histone and DNA. The crosslinking reaction was stopped by adding 0.125 M glycine (final concentration). Cells were washed twice with ice-cold PBS. The cells were resuspended in cell lysis buffer (5 mM PIPE, 85 mM KCl, 1% NP40, and protease inhibitors) and incubated on ice for 15 min. The nuclei were centrifuged at 5,000 rpm at 4°C for 5 min. The pellet of nuclei was resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitors) and incubated on ice for 10 min. The resulting solution was then sonicated and the lysate was centrifuged at 14,000 rpm at 4°C for 10 min. The supernatant was collected. Twenty-five microlitres of Strep A cells was added and incubated on a rotating platform for 15 min to preclear chromatin. The precleared chromatin was centrifuged at 14,000 rpm at 4°C for 4 min. Immuneprecipitation buffer including protease inhibitors was added to the collected precleared chromatin solution. Anti-WT1 (C-19) rabbit polyclonal antibody or anti-Sp1 rabbit polyclonal antibody was added into the solution. IgG and RNA polymerase II were used as positive and/or negative controls. The percleared chromatin lysate was rotated on a platform at 4°C overnight. Twenty-five microliters of Strep A cells was added to immunoprecipitated antibody/protein/DNA complexes and rotated on a platform for 15 min at room temperature to precipitate the complexes. The immunoprecipiated complex pellets were centrifuged at 14,000 rpm at room temperature for 4 min. The pellets were washed twice with dialysis buffer and four times with immuneprecipitation buffer. The immunoprecipiated complexes were then eluted with elution buffer (50 mM $NaHCO_3$, 1% SDS). The eluate was treated with 5 M NaCl and incubated at 65°C overnight to reverse formaldehyde crosslinking. RNase

was added (final concentration is 100 μg/mL) and the mixture was incubated at 37°C for 30 min. DNAs were purified by Qiaquick PCR Purification Kit (Qiagen, USA) and analysed by PCR.

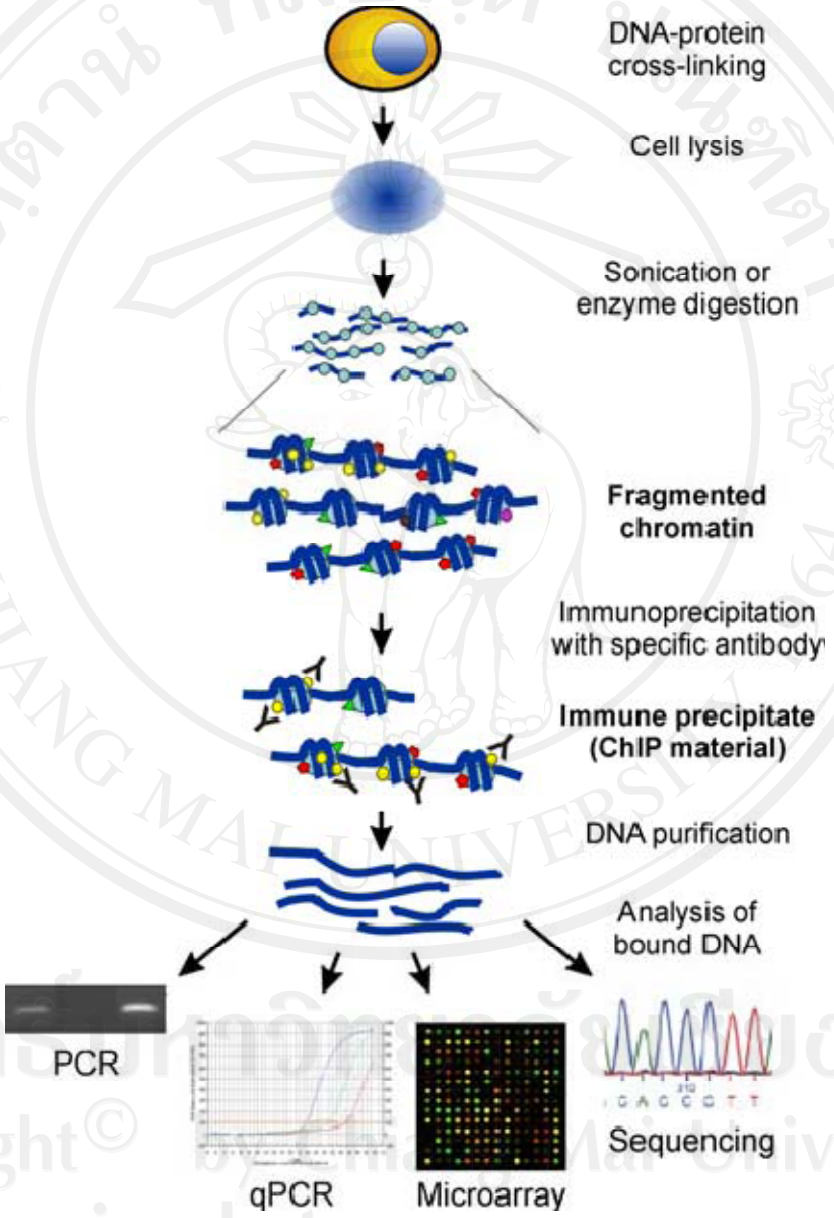

Figure 10 The chromatin immunoprecipitation (ChIP) assay and various methods of analysis (269).

2.21 Primer design for ChIP experiment

The sequence of the human WT1 promoter region was obtained from GenBank™ (accession No. U77682) and based on the DNA sequence published by Fraizer *et al*. (1994) (270). Primer design programs used in this study were Primer 3 Input (version 0.4.0), UCSC Genomic Bioinformatic and Vector NTI Advance 10. The primers of WT1 promoter were designed in each 500 bp. The WT1 promoter primer sequence No.1 consisted of forward primer CTGAACGGACTCTCCAGTG and reverse primer CGCTGCCTTGAACTCCTTAC. The WT1 promoter primer sequence No.2 consisted of forward primer GGCCCCTCTTATTTGAGCTT and reverse primer CAAGAGGAAGTCCAGGATCG.

2.22 Reporter gene assay

WT1 promoter construction vectors were kindly gifted by Professor Dr. Takashi Murate (Department of Medical Technology, Nagoya University Graduate School of Heath Sciences, Japan). The construct vector consisted of 1.8 kb-fragment covering the of 5' region of exon1 of the *WT1* gene promoter, including the WT1 binding site. The three construct vectors of -1807, -301, and -224 bp from TSS of exon1 of WT1 promoter were inserted into pGL3 basic vector. The pGL3 basic vector and renilla or beta-galactosidase vectors were used as vector control and internal control, respectively. K562 cells were co-transfected with the construct vector and renilla or bata-galactosidase for 24 h and then treated with 15 μM pure curcumin. The co-transfected cells were lysed by lysis buffer. Luciferase activity of the lysis solution was measured using a Dual-Luciferase Reporter Assay Kit (Promega, USA),

Beta-galactosidase Assay Kit (Promega, USA) and a GloMax 96 Microplate Luminometer with dual injections (Promega, USA).

2.23 Mutation promoter target assay

The QuikChange® Primer Design Program (QuikChangeII online primer design program) was used for mutation primer design. The target construct vector was used as a template for mutation PCR. The PCR mixture solution consisted of 25 ng of template, 1X *Pfu* turbo buffer, 4 mM dNTP, 2.5 ng/µL forward and reverse primer, 1 mM $MgSO_4$, 2% DMSO, 0.05 U/µL *Pfu* Turbo. PCR amplification was performed for 24 cycles of sequential denaturation (95°C, 30 sec); annealing (55°C, 60 sec) and extension (68°C, 5 min). After amplification, 10 µl of the PCR product was loaded into 1% agarose gel, to check the amplification results. Then 2 µL of 20 U *Dpn1* was added to the remaining 40 µL of PCR product and incubated at 37°C for 1 h to digest the parental template plasmid DNA. Ten microliters of digested plasmid DNA was transformed into highly-competent *E. coli* (Top10) and grown in LB broth with ampicillin. Plasmid was prepared and sent to sequence the construct mutation.