Cancer Chemother Pharmacol (2008) 62:585–594
DOI 10.1007/s00280-007-0642-1

ORIGINAL ARTICLE

Effect of pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin on *WT1* gene expression in leukemic cell lines

Songyot Anuchapreeda · Singkome Tima · Chadarat Duangrat · Pornngarm Limtrakul

Received: 27 July 2007 / Accepted: 31 October 2007 / Published online: 23 November 2007

Abstract
Purpose Leukemias are groups of hematological malignancies with high incidence and mortality rates in patients worldwide. There have been shown in many studies that *Wilms' tumor1* (*WT1*) gene were highly expressed in leukemic blast cells. Curcuminoids, major active components of the spice turmeric, are well known for its anticancer. Curcuminoids consist of pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin. In this study, the effect of each curcuminoids'components on *WT1* gene expression in leukemic cell lines (K562, HL60, U937, and Molt4) was investigated.
Methods The levels of WT1 mRNA and WT1 protein in leukemic cell lines were assessed by RT-PCR and Western blot analysis, respectively.
Results It was found that the WT1 mRNAs were detected in all 4 types of leukemic cell lines. However, the WT1 protein levels were found only in the cell lines K562 and Molt4. Pure curcumin exhibited a strong inhibitory effect on WT1 mRNA and WT1 protein expression. The treatment of leukemic cell lines with non-cytotoxic doses (5, 10, and 15 μM) of pure curcumin for 2 days reduced the level of WT1 mRNA expression and WT1 protein in a dose-dependent manner. In addition, pure curcumin at 10 μM significantly decreased the level of WT1 mRNA and protein in a time-dependent manner.
Conclusion Pure curcumin, an excellent curcuminoid derivative, decreased *WT1* gene expression in both transcriptional and translational levels. Thus, pure curcumin is one of a potential chemotherapeutic agent used for treatment of human leukemia. However, its chemotherapeutic property will need to be studied more in future.

Keywords Pure curcumin · Demethoxycurcumin · Bisdemethoxycurcumin · *WT1* gene · Leukemic cell lines

S. Anuchapreeda (✉) · S. Tima
Division of Clinical Microscopy,
Department of Medical Technology,
Faculty of Associated Medical Sciences,
Chiang Mai University, Chiang Mai, Thailand
e-mail: sanuchapreeda@yahoo.com

S. Tima
e-mail: singkome3@hotmail.com

C. Duangrat
Department of Pharmaceutical Sciences, Faculty of Pharmacy,
Chiang Mai University, Chiang Mai, Thailand
e-mail: chadarat@pharmacy.cmu.ac.th

P. Limtrakul
Department of Biochemistry, Faculty of Medicine,
Chiang Mai University, Chiang Mai, Thailand
e-mail: plimtrak@mail.med.cmu.ac.th

Introduction

Leukemia is a group of diseases involving the blood-forming organs, and is characterized by uncontrolled increase of white blood cells. Approximately 35,000 new cases of leukemia will be diagnosed in United States in 2006 [1]. The detection of overexpression in specific oncogenes or tumor suppressor genes provides information that is useful in the diagnosis of leukemia and prognosis of the disease. The overexpression of Wilms' tumor (WT1) protein in leukemia is one example.

The *Wilms' tumor1* (*WT1*) gene is involved in tumorigenesis. It is located on the human chromosome 11p13 [2, 3]. In normal tissue, WT1 is restricted to kidney, testis, ovary, spleen, hematopoietic precursors, and the mesothelial cell lining of visceral organs [4]. WT1 transcripts have been detected in adult bone marrow, lymph nodes, and

peripheral blood. These data suggest that WT1 may play a role in the development of erythroid, myeloid, and lymphoid cells during both the fetal and the adult stages. Previous studies demonstrated that the *WT1* gene is overexpressed in many types of cancer. It has been described as a tumor suppressor gene responsible for Wilms' tumor or nephroblastoma and other cancers [5–8]. However, recent studies have shown that *WT1* acts as an oncogene in many types of malignant tumors, especially leukemia [9]. It plays an important role in the progression of disease and prognosis of human malignancies [10, 11]. Significant levels of *WT1* gene expression have been found in leukemic samples; the average levels are approximately 1,000–100,000 times higher than in normal bone marrow and peripheral blood, respectively [10].

The *WT1* gene product is a 48–52 kDa nuclear protein that has four zinc fingers and acts as a transcriptional activator or repressor, depending on the cellular or chromosomal context [12, 13]. The four major isoforms of WT1 protein identified to date result from two alternative splices in its transcript. WT1 protein plays an important role in the development of normal cells and tissues. It may influence the proliferation and differentiation of blood cells by differential regulation of the genes for *TGFβ*, *C-Myc*, *C-Myb*, *Bcl2*, and the retinoic acid receptor [14]. Low levels of WT1 protein expression have been found in normal blood cells. In contrast, the overexpression of WT1 protein has been found in leukemic blood cells. The expression of WT1 in leukemic cells is involved in cell proliferation, differentiation, and apoptosis [14, 15]. Moreover, the expression of the *WT1* gene and its product have been used as biological markers for diagnosis and evaluation of the prognosis of leukemia and minimal residual disease (MRD) [10, 16]. All of these findings support the conclusion that *WT1* gene expression may be involved in leukemogenesis.

To date, chemotherapy has been the most frequently used treatment for leukemia. Chemotherapy works by destroying leukemic cells, hence stopping them from growing or multiplying. However, some normal cells are destroyed as well by this method of treatment. Due to the wide range of biological activities and lack of toxicity in animal models, natural remedies have been used as alternative treatments for leukemia and other malignant tumors.

Turmeric (*Curcuma longa* Linn) is one of the most popular herbs used for medical treatment, due to its wide variety of medicinal properties such as anti-inflammatory, anti bacterial, anti fungal, anti-oxidant, anticarcinogen, antimutagen, and anticancer properties. The active constituents of turmeric are known as curcuminoids, yellow pigmented substances isolated from the rhizome of turmeric. This active extract can be subdivided into three distinct components: pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Curcuminoids are a group of diet-derived agents that are being clinically evaluated as chemopreventive agents for major cancer targets, including the breast, prostate, lung, stomach, duodenal, and colon cancers, as well as leukemias [17, 18]. Curcuminoids, especially pure curcumin, have strong anticancer agent and anti-tumor progression properties [19–21].

The commercial grade curcuminoid mixture is commonly known as "curcumin" due to its main component being, curcumin. Curcumin has been found to induce cell death in two leukemic cell lines: K562 and Jurkat cells [22]. Moreover, the dietary component of pure curcumin induces apoptosis in human leukemia HL60 cells at the very low concentration of 3.5 μg/ml [23]. Recently, all three curcuminoids has been shown to have cytotoxic activity on leukemic cell lines HL60, U937, and K562 [24]. Moreover, the inhibitory effects of curcumin were associated with a decrease in the levels of *WT1* gene expression in patient leukemic cell and K562 cell lines [25, 26].

In this study, the effects of turmeric curcuminoids; curcumin, demethoxycurcumin, bisdemethoxycurcumin, commercial grade curcuminoid mixtures (Sigma-Aldrich), and in-house curcuminoid mixtures, on *WT1* gene expression in four leukemic cell lines: K562, U937, HL60, and Molt4 were investigated and compared.

Materials and methods

Cell culture

Human erythroid leukemia (K562), human promyeloid leukemia (HL60), human monocytic leukemia (U937), and human lymphoblastic leukemia (Molt4) were cultured as described elsewhere [25, 26].

Extraction and isolation of curcuminoids

Curcuminoid extraction and isolation were prepared as described previously [27, 28]. In this study, individual curcuminoids (pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin) has been purified from turmeric powder by column chromatography.

Western blotting

Cell nuclear extracts were prepared by NE-PER® Nuclear and Cytoplasmic Extraction Reagents (PIERCE, USA). The cell nuclear proteins (100 μg/lane) were separated by 12% SDS-polyacrylamide gel electrophoresis. WT1 protein detection was performed using primary rabbit polyclonal anti-WT1 clone C-19 (Santa Cruz, USA) at 1:1,000, followed by a treatment with HRP-conjugated goat anti-rabbit IgG (Promega, USA) at a 1:15,000 dilution. Then the same membrane was reprobed for GAPDH protein by Restore™

Western Blot Stripping Buffer (PIERCE, USA). After the stripping procedure, the nitrocellulose membrane was probed again with primary rabbit polyclonal anti-GAPDH clone FL-335 (PIERCE, USA) at a 1:1,000, followed by a treatment with HRP-conjugated goat anti-rabbit IgG (PIERCE, USA) at a 1:15,000 dilution. Proteins were visualized by the SuperSignal® protein detection kit (PIERCE, USA) and quantified by scan densitometer.

RNA extraction and quantitative RT-PCR

RNAs of leukemic cells were isolated by TRIZOL® reagent according to the manufacturer's instructions. RNaseOUT™ (Invitrogen, USA) was added to the RNA extraction products for RNA protection. RT-PCR was performed using SuperScript™ III One-step RT-PCR System with Platinum® *Taq* DNA polymerase reagent (Invitrogen, USA). For WT1, the sense primer was 5′-GGCATCTGAGACCAGTGAGAA-3′, and the antisense primer was 5′-GAGAGTCAGACTTGAAAGCAGT-3′, corresponding to residues 780–800 and residues 1,232–1,253, respectively, of the published cDNA sequence [29]. cDNA was synthesized from 1 μg of total RNA at 60°C for 30 min and denatured at 94°C for 2 min. PCR amplification was performed for 30 cycles of sequential denaturation (94°C, 1 min); annealing (60°C, 1 min); and extension (72°C, 1 min), which yields gave a 474-bp product. For GAPDH, the sense primer (5′-CGAAGTCAACGGATTTGGTCGTAT-3′) and the anti-sense primer (5′-AGCCTTCTCGGTGGTGAAGAC-3′) were used. These sequences were corresponding to residues 888–911 and residues 1,174–1,194, respectively. The GAPDH cDNA was synthesized from 1 μg of total RNA at the same condition of WT1, which yield a 306-bp product. For the negative control, water was amplified a total of 30 cycles to detect possible contamination. A total 15 μl of each PCR product was analyzed via 1% agarose gel electrophoresis, visualized with ethidium bromide staining (2 mg/ml), and quantified using scan densitometry (BIO-RAD, Richmond, CA, USA).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD) from triplicate samples of three independent experiments. Statistical differences between the means were tested one-way ANOVA. Probability values $P < 0.05$ were considered to be statistically significant.

Results

WT1 mRNA and WT1 protein were detected in K562, U937, HL60, and Molt 4 cell lines. However, the WT1 protein levels were found only in K562 and Molt4 cells (Fig. 1a, b). To determine the effect of three turmeric curcuminoids, including commercial grade curcuminoid (Sigma-Aldrich) and in-house curcuminoids on *WT1* gene expression, all leukemic cell lines were treated with a non-cytotoxic concentration (10 μM) of curcuminoids for 2 days. All curcuminoid derivatives decreased the WT1 mRNA levels and WT1 protein levels in leukemic cell lines. The levels of WT1 mRNA (after normalization to GAPDH expression) in K562 cells were decreased by 59, 29, 39, 54, and 37% (Fig. 2a, e), in U937 cells by 43, 18, 28, 25, and 8% (Fig. 2b, e), in HL60 cells by 37, 14, 15, 16, and 7% (Fig. 2c, e), and in Molt4 cells by 17, 3, 3, 2, and 0.8% (Fig. 2d, e). The levels of WT1 protein in K562 cells were decreased by 48, 13, 42, 40, and 22% (Fig. 3a, c), and in Molt4 cells were 90, 57, 68, 30, and 26% (Fig. 3b, c) in response to treatment with pure curcumin, demethoxycurcumin, bisdemethoxycurcumin, commercial grade curcuminoid mixture (Sigma-Aldrich), and curcuminoid mixture (in-house curcuminiods), respectively, compared with the vehicle control. However, pure curcumin exhibited a strong inhibitory effect on WT1 mRNA and protein levels in K562, U937, HL60, and Molt4.

To verify that pure curcumin could modulate the spontaneous WT1 expression (mRNA) occurring in vitro, as well as WT1 protein expression, Leukemic cells were treated with pure curcumin (5, 10, and 15 μM) for 2 days. Levels of WT1 mRNA expression in K562 cells were decreased by 15, 28 and 50% (Fig. 4a, e), in U937 cells by 10, 17, and 19% (Fig. 4b, e), in HL60 cells by 5, 11, and 18% (Fig. 4c, e) and in Molt 4 cells by 6, 19 and 21% (Fig. 4d, e) in

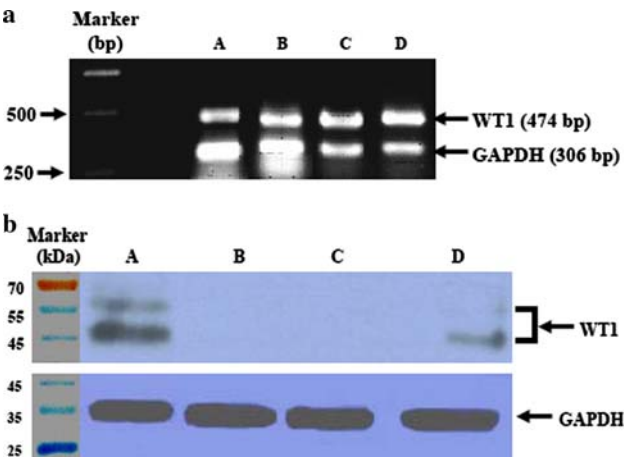

Fig. 1 The WT1 mRNA (**a**) and protein (**b**) levels in K562, U937, HL60 and Molt4 cell lines. The WT1 and GAPDH mRNA levels in untreated K562 (*A*), U937 (*B*), HL60 (*C*), and Molt4 (*D*) cell lines were determined by RT-PCR. Fifteen microlitres of the PCR products (474 bp for WT1 and 306 bp for GAPDH) were run on 1% agarose gel. The WT1 protein level was determined by Western blot analysis using rabbit polyclonal anti-WT1 antibody (*WT1*; *C-19*). The level of GAPDH protein was used as an internal control

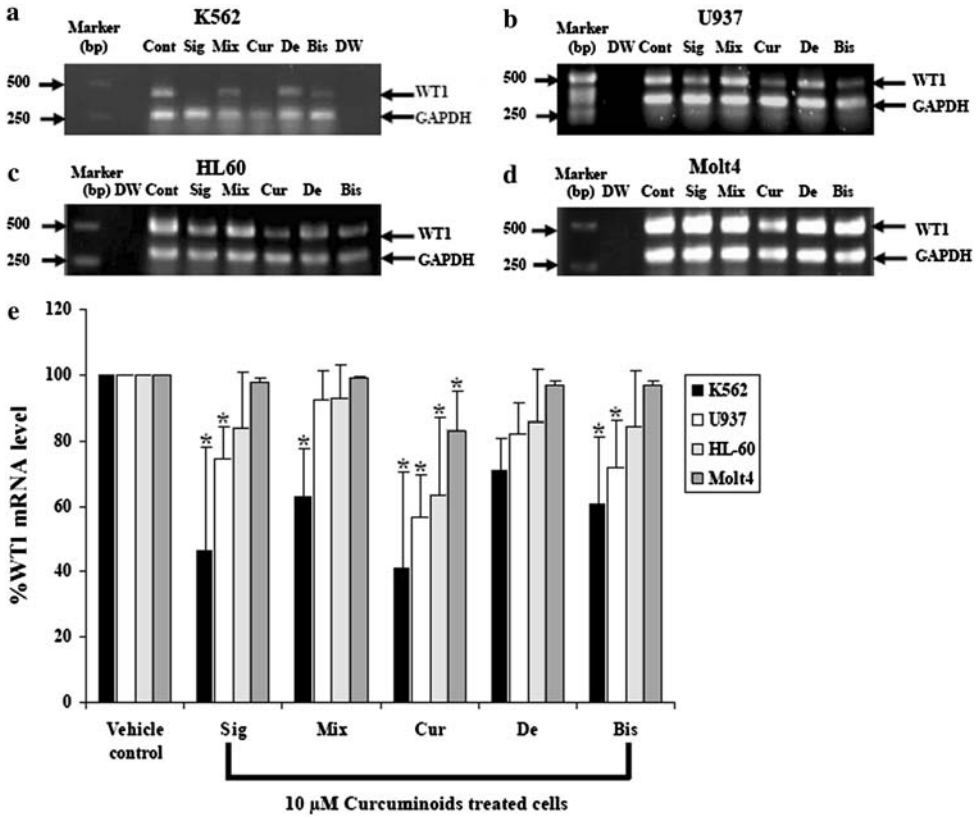

Fig. 2 Analysis of the effect of curcuminoid extracts on WT1 mRNA level in K562, U937, HL60, and Molt4 cell lines. The WT1 and GAPDH mRNA levels following treatment with 0.05% DMSO (Cont; Control) and 10 μM of commercial grade curcuminoid mixture (Sig; Sigma-Aldrich), curcuminoid mixture (*Mix*), pure curcumin (*Cur*), demethoxycurcumin (*De*), and bisdemethoxycurcumin (*Bis*) were determined in K562 (**a**), U937 (**b**), HL60 (**c**), and Molt4 (**d**) cells after 2 days by RT-PCR. Fifteen microlitres of the PCR products (474 bp for WT1 and 306 bp for GAPDH) were run on 1% agarose gel. The bands were quantified using a scan densitometer (**e**). WT1 mRNA levels were measured and normalized with GAPDH mRNA level. Distilled water (*DW*) was used as a negative control. Data are the mean values ± standard deviation (SD) of three independent experiments. *Asterisks* (*) denote values that were significantly different from the vehicle control ($P < 0.05$)

response to treatment with 5, 10, and 15 μM pure curcumin, respectively, compared with the vehicle control. WT1 protein levels in K562 cells were decreased by 17, 37, and 81% (Fig. 5a, c) and in Molt4 cell line by 56, 79, and 94% (Fig. 5b, c) in response to treatment with 5, 10, and 15 μM pure curcumin, respectively.

Pure curcumin significantly decreased *WT1* expression at the mRNA and protein levels. Therefore, subsequent experiments were designed to observe modulating effects of pure curcumin on *WT1* expression at various incubation times. Expression of WT1 mRNA in K562 cells after treatment for 1, 2, and 3 days with pure curcumin was decreased by 37, 48, and 54% (Fig. 6a, e), in U937 cells by 13, 33, and 64% (Fig. 6b, e), in HL60 cells by 4, 8, and 30% (Fig. 6c, e) and in Molt4 cells by 7, 11, and 16% (Fig. 6d, e), respectively, compared with the vehicle control. WT1 protein levels in K562 cells were decreased by 50, 92, and 99% (Fig. 7a, c) and in Molt4 cell line by 53, 84, and 92% (Figs. 7b, 6c) in response to treatment with 10 μM pure curcumin for 1, 2, and 3 days, respectively. To investigate the rate of proliferation after incubating leukemic cells with various concentrations of pure curcumin (0, 5, 10, and 15 μM), the number of leukemic cells was determined at 0, 1, 2, and 3 days after incubation using a trypan blue dye exclusion assay. The proliferation rates of K562 cells were decreased by 25, 30, and 49% (Fig. 8a), the rates of Molt4 cell proliferation were decreased by 13, 32, and 42% (Fig. 8b), the proliferation rates of U937 cells were decreased by 31, 33, and 38% (Fig. 8c) and the proliferation rates of HL60 cells were decreased by 22, 33, and 64% (Fig. 8d) when treated with 5, 10, and 15 μM pure curcumin, respectively (compared to control without curcumin treatment).

Discussion

Leukemia is a cancerous disorder of the blood-forming tissues, especially bone marrow. It is characterized by excessive production of immature or mature leukocytes and

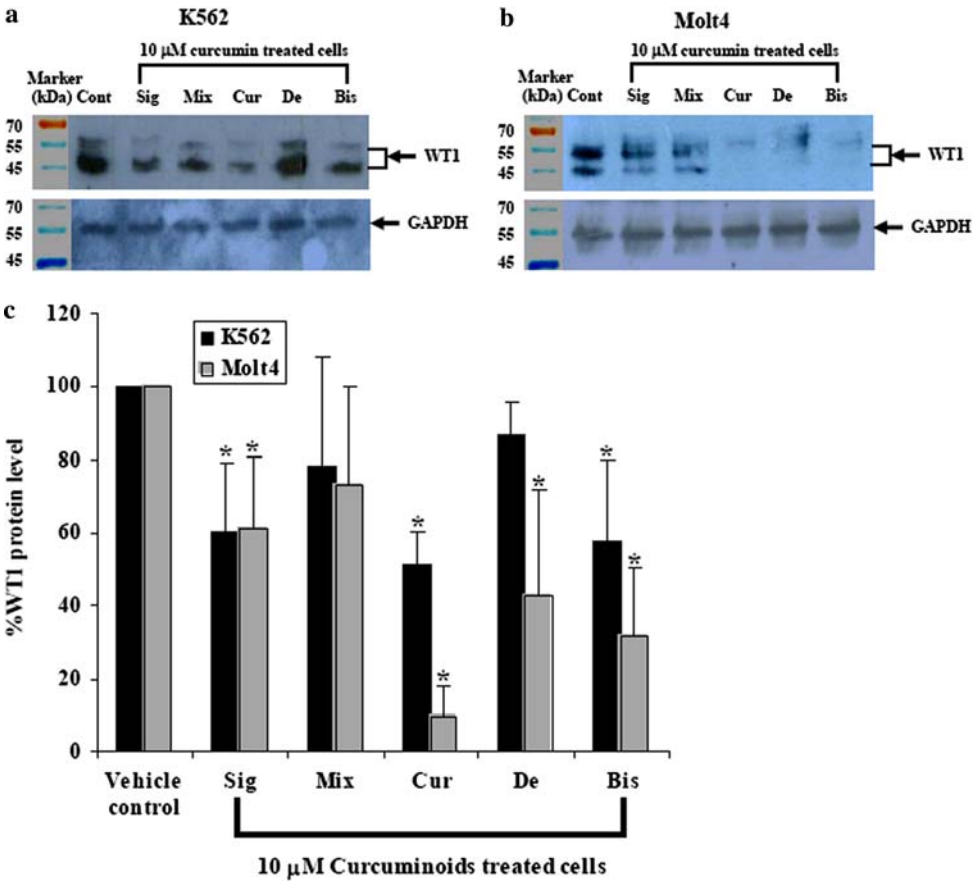

Fig. 3 Analysis of the effect of curcuminoid extracts on WT1 protein level in K562 and Molt4 cell lines. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO (Cont; Control) and 10 μM of commercial grade curcuminoid mixture (Sig; Sigma-Aldrich), curcuminoid mixture (*Mix*), pure curcumin (*Cur*), demethoxycurcumin (*De*), and bisdemethoxycurcumin (*Bis*) were determined in K562 (**a**) and Molt4 (**b**) cells after 2 days by Western blot analysis. The bands (48–54 kDa for WT1 and 37 kDa for GAPDH were quantified using a scan densitometer (**c**). WT1 protein levels were measured and normalized with GAPDH protein level. Data are the mean value ± standard deviation (SD) of three independent experiments. *Asterisks* (*) denote values that were significantly different from the vehicle control ($P < 0.05$)

consequently a crowding-out of red blood cells and platelets. *Wilms' tumor1* (*WT1*) gene is involved in leukemogenesis. Previous studies have shown that the wild-type *WT1* gene is strongly expressed in leukemic blast cells with an increase in its expression levels at relapse and inverse correlation between its expression levels and prognosis [10, 30–32]. In addition, *WT1* gene has been shown to be overexpressed in leukemic cells [33]. These findings suggest that *WT1* plays an important role in leukemogenesis and may have an oncogenic function rather than a tumor-suppressive function in hematopoietic progenitor cells and leukemic blast cells.

In anticancer drug research, dietary and/or medicinal plants, such as turmeric, ginger, garlic, chili, and pepper are becoming popular as chemotherapeutic drugs. Turmeric is a spice grown in tropical regions of Asia. Curcuminoids have been identified as the major active yellow pigment in turmeric and consist of three major active ingredients; pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin. These three curcuminoid compounds are always mixed together in turmeric powder as found in commercial grade curcuminoids. Curcuminoids have a wide range of biological and pharmacological activities, including antioxidant and anti-inflammatory effects, as well as anti-mutagen and anticancer properties [19–21, 34]. The expression of STAT5 mRNA and protein in K562 cells was inhibited by curcumin, as well as K562 cell proliferation [35]. Moreover, the expression of several oncogenes such as *c-jun* and NF_kB are suppressed by curcumin [36, 37]. Recently, commercial grade curcuminoids (Sigma-Aldrich) have shown suppressive effects on the expression of WT1 mRNA and WT protein levels in human leukemic cells [25, 26]. However, there has been to date no indication of which component of turmeric has the strongest suppressive effect on *WT1* gene and protein expression in leukemic cell lines.

The WT1 mRNA was detected in four types of leukemic cell lines. The *WT1* gene was overexpressed in all types of leukemic cells. This supports the conclusions from previous studies, that the *WT1* gene plays a role in leukemogenesis and may have an oncogenic function rather than a tumor-suppressor gene function in leukemic cells [9, 14, 38]. Conversely, high levels of WT1 protein were detected in K562 and Molt4 cell lines. Possibly the WT1 protein levels in U937 and HL60 are too low to be detected by our methods.

After being treated with 10 μM curcuminoids (non-cytotoxic dose), the morphology and viability of the leukemic cells did not change when compared to those of the vehicle control (data not shown). This suggests that curcuminoid extracts at a concentration of 10 μM do not alter the morphology and viability of leukemic cell lines. In addition,

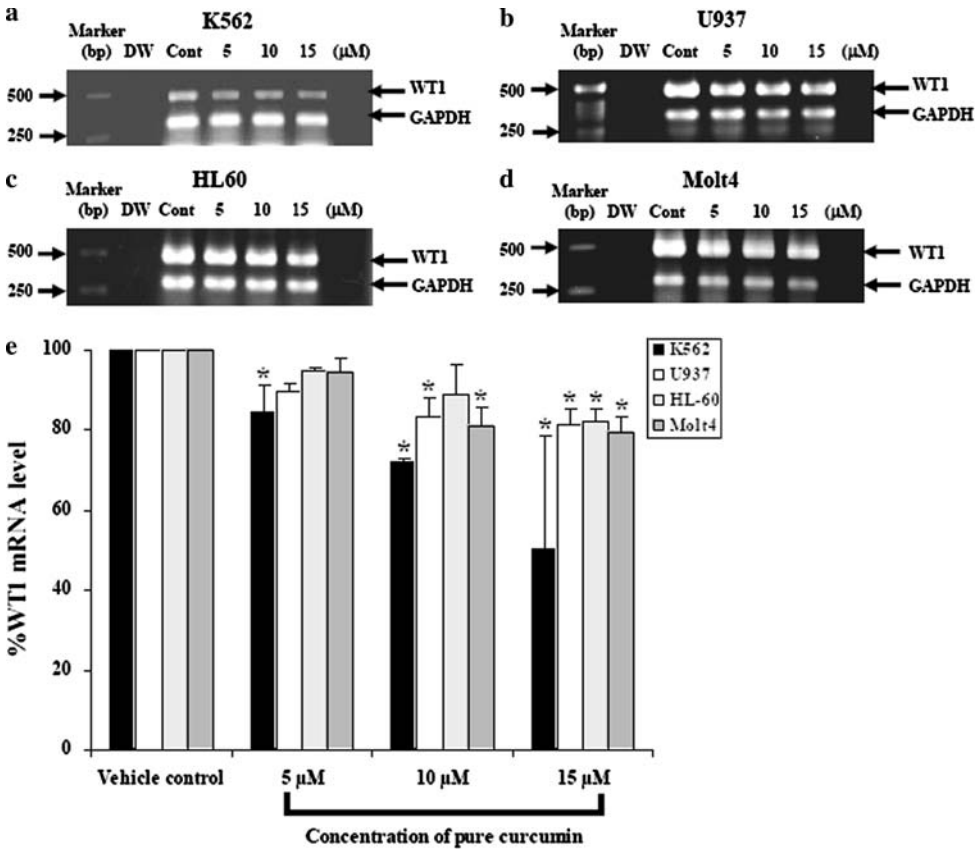

Fig. 4 The WT1 mRNA levels in K562, U937, HL60, and Molt4 cell lines cultured in 5, 10, and 15 μM of pure curcumin for 2 days. The WT1 and GAPDH mRNA levels following treatment with 0.05% DMSO (Cont; Control) and pure curcumin at a concentration 5, 10, and 15 μM were determined in K562 (**a**), U937 (**b**), HL60 (**c**), and Molt4 (**d**) cells after 2 days by RT-PCR. Fifteen microlitres of the PCR products were electrophoresed on 1% agarose gel. The bands were quantified using a scan densitometer (**e**). WT1 mRNA level was measured and normalized with GAPDH mRNA level. Distilled water (*DW*) was used as a negative control. Data are the mean value ± SD of three independent experiments. *Asterisks* (*) denote values that were significantly different from the vehicle control ($P < 0.05$)

WT1 mRNA and WT1 protein levels in leukemic cell lines were decreased by all curcuminoid derivatives, including commercial grade curcuminoids, in-house curcuminoid mixture, pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin after normalization. Moreover, the results clearly show that pure curcumin, the major component of curcuminoid powder, is the most effective component in decreasing WT1 mRNA and WT1 protein levels, followed in decreasing order by bisdemethoxycurcumin, and demethoxycurcumin. This result is similar to that of Nagabhushan [19]. Pure curcumin affected cell proliferation rates of four leukemic cell lines due to the suppression of *WT1* gene expression.

The vanillyl (4-hydroxy-3-methoxyphenyl) moiety and a ketone functional group in the structure of [6]-Gingerol and [6]-paradol, which share common structural features found in curcuminoid, induce HL60 cell death via cell apoptosis [39]. Moreover, the diketone system of curcuminoids appears to be the part of the molecule involved in the antiproliferative effect of curcuminoids [40]. Both phenolic hydroxyl groups, phenolic methoxyl groups, and the diketone moiety could be responsible for the inhibitory effect on *WT1* gene expression. The present study suggests that hydroxyl groups on the benzene rings are not essential for this activity, because various curcuminoid derivatives have the same number of hydroxyl groups but show different levels of inhibitory effect. The present results are consistent with the hypothesis that the functional groups, which play a role in the inhibitory effect on *WT1* gene expression are phenolic methoxyl groups of each curcuminoid derivative. Pure curcumin, which possesses two methoxyl groups, exhibited the maximum inhibitory effect, while demethoxycurcumin and bisdemethoxycurcumin, which possess one and no methoxyl group, respectively, were less inhibitory. In addition, an imbalance between the methoxyl groups on the structure of demethoxycurcumin may influence this activity. This study suggests that the diketone groups and the imbalance of the phenolic methoxyl groups of curcuminoids may contribute to the inhibitory effect of curcuminoids on *WT1* gene expression.

The effect of pure curcumin on *WT1* gene expression in leukemic cell lines was evaluated. It was found that pure curcumin significantly decreased the WT1 mRNA and WT1 protein levels in dose and time-dependent manners. A decrease in *WT1* gene expression with increasing pure curcumin concentrations (5–15 μM), demonstrated that pure curcumin inhibited the level of immunoreactive WT1 protein observed in K562 and Molt4 cells and also reduced the level of WT1 mRNA under the same conditions in four types of leukemic cell lines used in this study. Furthermore, the experiments also showed that treatment of cells with pure curcumin at 10 μM for 1–3 days inhibited the *WT1*

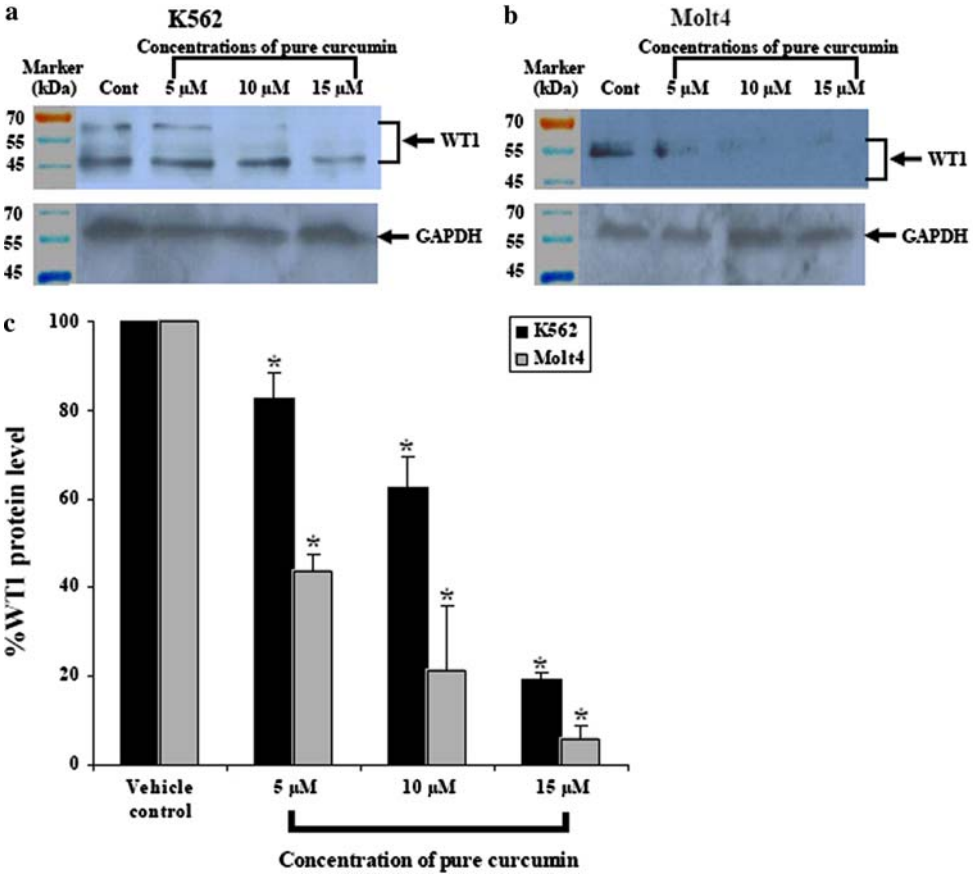

Fig. 5 The WT1 protein level in K562 and Molt4 cell lines cultured in 5, 10, and 15 μM of pure curcumin for 2 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO (Cont; Control) and pure curcumin at concentrations of 5, 10, and 15 μM were determined in K562 (**a**) and Molt4 (**b**) cells after 2 days by Western blot analysis. The bands (48–54 kDa for WT1 and 37 kDa for GAPDH were quantified using a scan densitometer (**c**). WT1 protein levels were measured and normalized with GAPDH protein level. Data are the mean value ± standard deviation (SD) of three independent experiments. *Asterisks* (*) denote values that were significantly different from the vehicle control ($P < 0.05$)

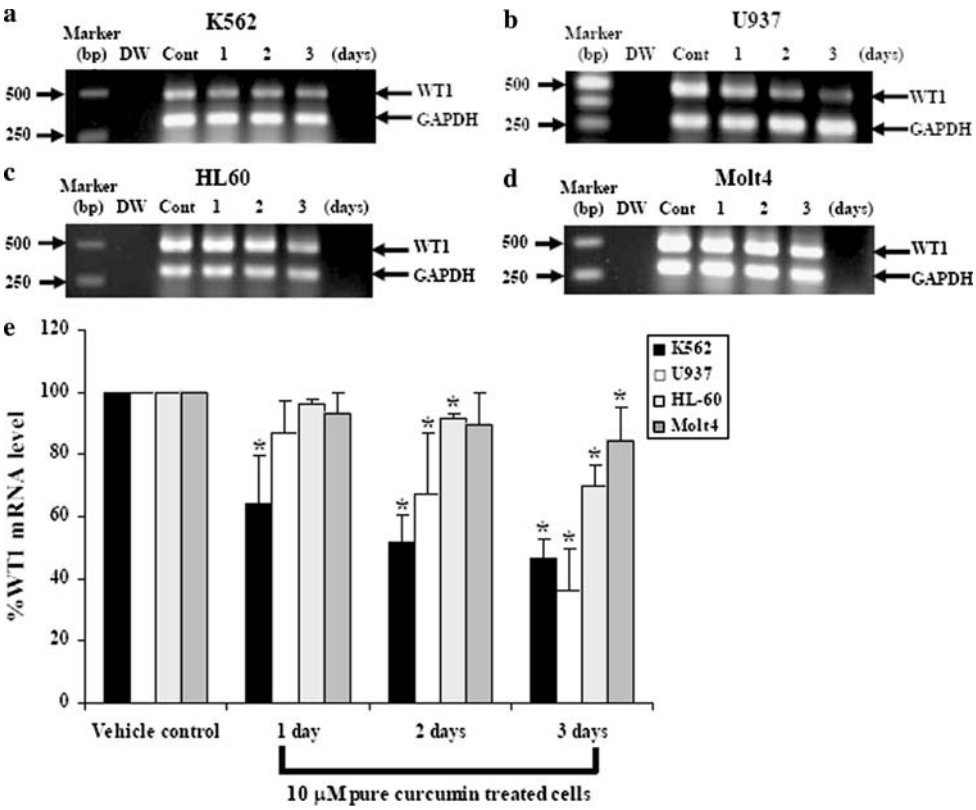

Fig. 6 The WT1 mRNA levels in K562, U937, HL60, and Molt4 cell lines cultured in 10 μM of pure curcumin for 1, 2, and 3 days. The WT1 and GAPDH mRNA levels following treatment with 0.05% DMSO (Cont; Control) for 3 days and 10 μM pure curcumin for 1, 2, and 3 days were determined in K562 (**a**), U937 (**b**), HL60 (**c**), and Molt4 (**d**) by RT-PCR. Fifteen microlitres of the PCR products were electrophoresed on 1% agarose gel. The bands were quantified using a scan densitometer (**e**). WT1 mRNA level was measured and normalized with GAPDH mRNA level. Distilled water (*DW*) was used as a negative control. Data are the mean value ± SD of three independent experiments. *Asterisks* (*) denote values that were significantly different from the vehicle control ($P < 0.05$)

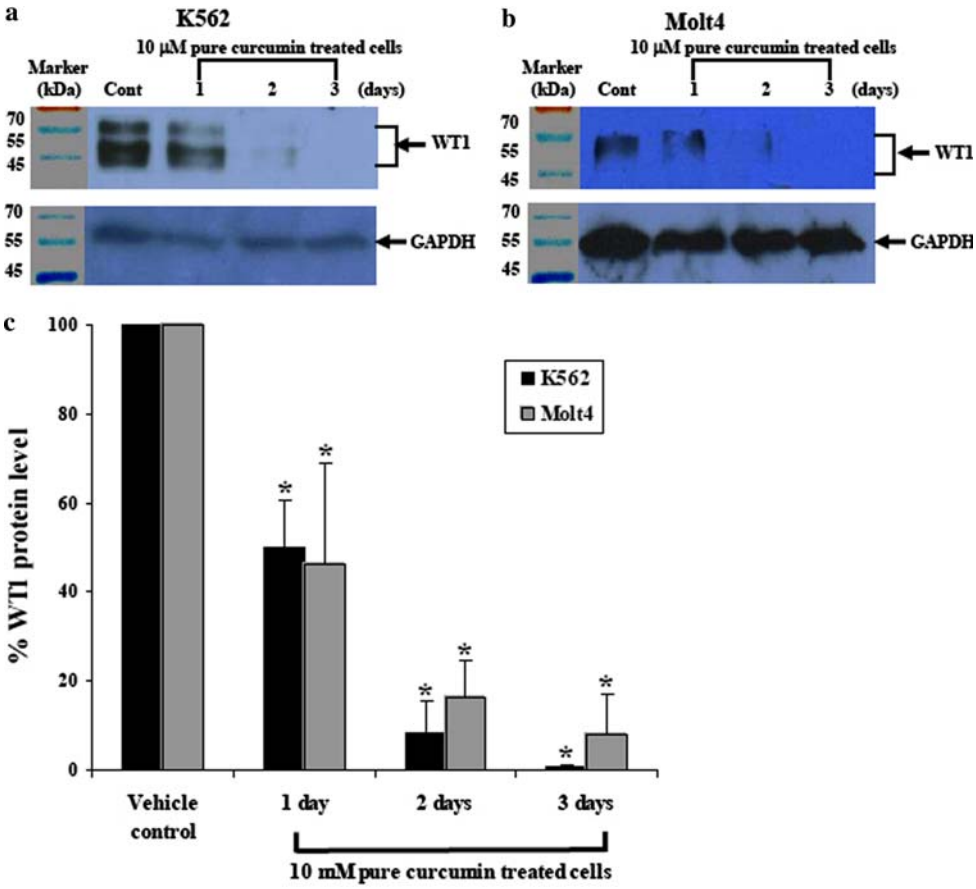

Fig. 7 The WT1 protein level in K562 and Molt4 cell lines cultured in 10 μM of pure curcumin for 1, 2, and 3 days. The WT1 and GAPDH protein levels following treatment with 0.05% DMSO (Cont; Control) and 10 μM of pure curcumin for 1, 2, and 3 days were determined in K562 (**a**) and Molt4 (**b**) cells by Western blot analysis. The bands (48–54 kDa for WT1 and 37 kDa for GAPDH were quantified using a scan densitometer (**c**). WT1 protein levels were measured and normalized with GAPDH protein level. Data are the mean value ± standard deviation (SD) of three independent experiments. *Asterisks* (*) denote values that were significantly different from the vehicle control ($P < 0.05$)

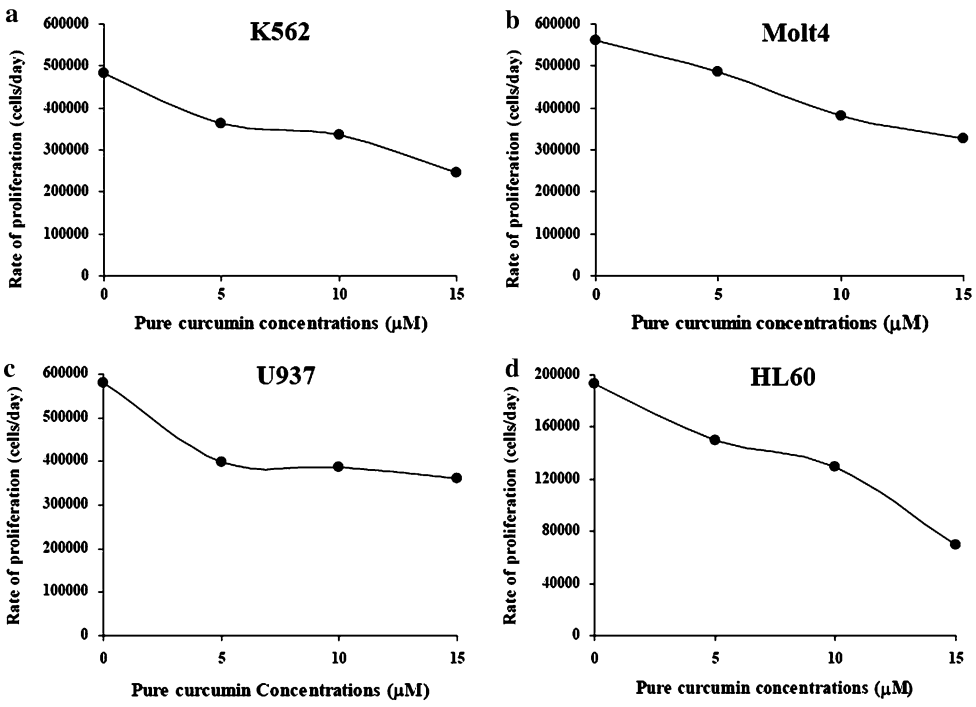

Fig. 8 The proliferation rate of K562 (**a**), Molt4 (**b**), U937 (**c**), and HL60 (**d**) cell lines cultured in 5, 10, and 15 μM of pure curcumin for 1, 2, and 3 days. After incubating cells with various concentrations of pure curcumin (0, 5, 10 and 15 μM), the numbers of leukemic cells were determined after 0, 1, 2, and 3 days via a trypan blue dye exclusion assay. The slope or cell growth rate at each predetermined pure curcumin concentration was determined from the plot of incubating time and cell number. Consequently, the rates of cell growth at different pure curcumin concentrations were compared with the normal growth rate, which was obtained from the control wells (no pure curcumin)

gene expression in a time-dependent manner. The changes in WT1 protein levels of Molt4 cells were more prominent compared to the mRNA levels. This could be explained in two ways: (a) via the effect of pure curcumin on mRNA stability and (b) by the effect of pure curcumin on post-transcription processing. Previous studies have suggested that

compounds with antioxidant or anti-inflmmatory activites also inhibit tumor promotion and cell proliferation. Pure curcumin is one of the compounds with remarkable antioxidant and free radical-scavenging activities [41–44]. Thus, pure curcumin also has an inhibitory effect on tumor promotion and cell proliferation. In a previous study pure curcumin was found to be the most effective in the DNA cleavage reaction and reduction of Cu (II) [45]. The presence of the diketone moiety in the pure curcumin molecule seems to be essential for the inhibitory activity [40].

Taken as a whole, the results indicate that treatment of human leukemic cell lines with non-cytotoxic concentrations (low doses; cell survival is higher than 80%) of pure curcumin inhibited *WT1* gene expression, whereas pure curcumin, at high dose, induced cell cytotoxicity (IC50 = 44 μM). In an additional study, the three curcuminoid compounds also showed cytotoxicity to K562, U937, and HL60 cell lines [25]. The current results suggest that pure curcumin can potentially be used as a chemotherapeutic agent in human leukemic cancer. This research may lead to clinical trials in the future.

Acknowledgments This work was supported by grants from the National Research Council of Thailand, Chiang Mai University Research Fund, the National Science and Technology Development Agency (NSTDA) of Thailand, Biomedical Technology Research Center, and Faculty of Associated Medical Sciences Endowment Fund. We gratefully acknowledge Dr. Dale E. Taneyhill, Mr. Greg Sholly for their critical reading of the manuscript.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ (2006) Cancer statistics CA. Cancer J Clin 56:106–130
2. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, Housman DE (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60:509–520
3. Gessler M, Konig A, Bruns GA (1992) The genomic organization and expression of the WT1 gene. Genomics 12:807–813
4. Buckler AJ, Pelletier J, Haber DA, Glaser T, Housman DE (1991) Isolation, characterization, and expression of the murine Wilms' tumor gene (WT1) during kidney development. Mol Cell Biol 11:1707–1712
5. Rauscher FJ III (1993) The WT1 Wilms tumor gene product: a developmentally regulated transcription factor in the kidney that functions as a tumor suppressor. FASEB J 7:896–903
6. Lewis WH, Yeger H, Bonetta L, Chan HS, Kang J, Junien C, Cowell J, Jones C, Dafoe LA (1988) Homozygous deletion of a DNA marker form chromosome 11p13 in sporadic Wilms' tumor. Genomics 3:25–31
7. Kikuchi H, Akasaka Y, Nagai T, Umezawa A, Iri H, Kato S, Hata J (1992) Genomic changes in the WT gene (WT1) in Wilms' tumors and their correlation with histology. Am J Pathol 140:781–786
8. Miyoshi Y, Ando A, Egawa C, Taguchi T, Tamaki Y, Tamaki H, Sugiyama H, Noguchi S (2002) High expression of Wilms' tumor suppressor gene predicts poor prognosis in breast cancer patients. Clin Cancer Res 8:1167–1171
9. Sugiyama H (2001) Wilms' tumor gene WT1: its oncogenic function and clinical application. Int J Hematol 73:177–187
10. Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T, Nasu K, Kyo T, Dohy H, Nakauchi H, Ishidate T, Akiyama T, Kishimoto T (1994) WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood 84:3071–3079
11. Bergmann L, Miething C, Maurer U, Brieger J, Karakas T, Weidmann E, Hoelzer D (1997) High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood 90:1217–1225
12. Wang ZY, Qiu QQ, Enger KT, Deuel TF (1993) A second transcriptionally active DNA-binding site for the Wilms tumor gene product, WT1. Proc Natl Acad Sci USA 90:8896–8900
13. Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T (1990) Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science 250:1259–1262
14. Menke AL, van der Eb AJ, Jochemsen AG (1998) The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? Int Rev Cytol 181:151–212
15. Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, Reinhardt R, Thiel E (1995) Presence of Wilms' tumor gene (WT1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. Leukemia 9:1060–1067
16. Guo X, Xu S, Dong Z (1999) WT1 gene expression in leukemia patients and its correlation with prognosis and multidrug resistance. Zhonghua Xue Ye Xue Za Zhi 20:69–72
17. Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW, Kopelovich L, Hawk ET, Lieberman R, Lawrence JA, Ali I, Viner JL, Sigman CC (2000) Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. J Nutr 130:467S-471S
18. Duvoix A, Blasius R, Delhalle S, Schnekenburger M, Morceau F, Henry E, Dicato M, Diederich M (2005) Chemopreventive and therapeutic effects of curcumin. Cancer Lett 223:181–190
19. Nagabhushan M, Bhide SV (1992) Curcumin as an inhibitor of cancer. J Am Coll Nutr 11: 192–198
20. Huang MT, Wang ZY, Georgiadis CA, Laskin JD, Conney AH (1992) Inhibitory effects of curcumin on tumor initiation by benzo(a)pyrene and 7,12 dimethylbenz (a) antracene. Carcinogenesis 13:2183–2186
21. Azuine MA, Bhide SV (1992) Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. Nutr Cancer 17:77–83
22. Duvoix A, Morceau F, Schnekenburger M, Delhalle S, Galteau MM, Dicato M, Diederich M (2003) Curcumin induced cell death in two leukemia cell lines: K562 and Jurkat. Ann NY Acad Sci 1010:389–392
23. Kuo ML, Huang TS, Lin JK (1996) Curcumin, an antioxidant and anti-tumor promoter, induces apoptosis in human leukemia cells. Biochim Biophys Acta 1317:95–100
24. Anuchapreeda S, Sadjapong W, Duangrat C, Limtrakul P (2006) The cytotoxic effect of curcumin, demethoxycurcumin and bisdemethoxycurcumin purified form turmeric powder on luekemic cell lines. Bull Chiang Mai Assoc Med Sci 39:60–71
25. Anuchapreeda S, Limtrakul P, Thanarattanakorn R, Sittipreechacharn S, Chanarat P (2006) Inhibitory effect of curcumin on WT1 gene expression in patient leukemic cells. Arch Pharm Res 29:80–87
26. Anuchapreeda S, Thanarattanakorn R, Sittipreechacharn S, Chanarat P, Limtrakul P (2006) Curcumin inhibits WT1 gene expression in human leukemic K562 cells. Acta Pharmacol Sin 27:360–366
27. Limtrakul P, Anuchapreeda S, Buddhasukh D (2004) Modulation of human multidrug-resistance MDR-1 gene by natural curcuminoids. BMC Cancer 4:1–6

28. Chearwae W, Anuchapreeda S, Nandigama K, Ambudkar SV, Limtrakul P (2004) Biochemical mechanism of modulation of human P-glycoprotein (ABCB1) by curcumin I, II, and III purified from Turmeric powder. Biochem Pharmacol 68:2043–2052
29. Oji Y, Ogawa H, Tamaki H, Oka Y, Tsuboi A, Kim EH, Soma T, Tatekawa T, Kawakami M, Asada M, Kishimoto T, Sugiyama H (1999) Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. Jpn J Cancer Res 90:194–204
30. Inoue K, Ogawa H, Yamagami T, Soma T, Tani Y, Tatekawa T, Oji Y, Tamaki H, Kyo T, Dohy H, Hiraoka A, Masaoka T, Kishimoto T, Sugiyama H (1996) Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. Blood 88:2267–2278
31. Inoue K, Ogawa H, Sonoda Y, Kimura T, Sakabe H, Oka Y, Miyake S, Tamaki H, Oji Y, Yamagami T, Tatekawa T, Soma T, Kishimoto T, Sugiyama H (1997) Aberrant overexpression of the Wilms tumor gene (WT1) in human leukemia. Blood 89:1405–1412
32. Tamaki H, Ogawa H, Inoue K, Soma T, Yamagami T, Miyake S, Oka Y, Oji Y, Tatekawa T, Tsuboi A, Tagawa S, Kitani T, Aozasa K, Kishimoto T, Sugiyama H, Miwa H, Kita K (1996) Increased expression of the Wilms tumor gene (WT1) at relapse in acute leukemia. Blood 88:4396–4398
33. Wu Y, Fraizer GC, Saunders GF (1995) GATA-1 transactivates the WT1 hematopoietic specific enhancer. J Biol Chem 270:5944–5949
34. Lantz RC, Chen GJ, Solyom AM, Jolad SD, Timmermann BN (2005) The effect of turmeric extracts on inflammatory mediator production. Phytomedicine 12:445–452
35. Chen WH, Chen Y, Gu JX, He J (2004) Effect of curcumin on STAT5 signaling molecule in K562 cells. Chin J Hematol 25:151–153
36. Huang TS, Lee SC, Lin JK (1991) Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. Proc Natl Acad Sci USA 88: 5292–5296
37. Pan MH, Lin-Shiaut SY, Lin JK (2000) Comparative studies on the suppression of nitric oxide synthase by curcumin and its hydrogenated metabolites through down-regulation of IkB kinase and NFkB activation in macrophage. Biochem Pharmacol 60:1665–1676
38. Inoue K, Tamaki H, Ogawa H, Oka Y, Soma T, Tatekawa T, Oji Y, Tsuboi A, Kim EH, Kawakami M, Akiyama T, Kishimoto T, Sugiyama H (1998) Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. Blood 91:2969–2976
39. Lee E, Surh YJ (1998) Induction of apoptosis in HL60 cells by pungent vanilloids, [6]-gingerol and [6]-paradol. Cancer Lett 134:163–138
40. Simon A, Allais DP, Duroux JL, Basly JP, Durand-Fontanier S, Delage C (1998) Inhibitory effect of curcuminoids on MCF-7 cell proliferation and structure-activity relationships. Cancer Lett 129:111–116
41. Kunchandy E, Rao MNA (1990) Oxygen radical scavenging activity of curumin. Int J Pharm 58:237–240
42. Soudamini KK, Kuttan R (1989) Inhibition of chemical carcinogenesis by curcumin. J Ethnopharmacol 27:227–233
43. Toda S, Miyase T, Arichi H, Tanizawa H, Takino Y (1985) Natural antioxidants. III. Antioxidative components isolated from rhizome of *Curcuma longa* L. Chem Pharm Bull 33:1725–1728
44. Ramsewak RS, DeWitt DL, Nair MG (2000) Cytotoxicity, antioxidant and anti-inflammatory activities of curcumins I-III from *Curcuma longa*. Phytomedicine 7:303–308
45. Ahsan H, Parveen N, Khan NU, Hadi SM (1999) Pro-oxidant, anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. Chem Biol Interact 121:161–175