Curcumin Suppresses Vasculogenic Mimicry Capacity of Hepatocellular Carcinoma Cells through STAT3 and PI3K/AKT Inhibition

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Abstract. *Background: Vasculogenic mimicry (VM) refers to the process in which highly invasive cancer cells mimic endothelial cells by forming blood channels. In the present study, we investigated the effect of curcumin, a natural product from turmeric, on VM of SK-Hep-1 human hepatocellular carcinoma (HCC) cells. Materials and Methods: In vitro VM, cell migration, and matrix metalloproteinase-9 (MMP9) production of HCC cells were determined by Matrigel tube formation assay, Transwell cell migration assay, and gelatin zymography, respectively. Effects of curcumin on AKT, signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK) and nuclear factor-κB (NF-κB) signaling pathways were determined by immunoblot analysis. Results: At non-cytotoxic concentrations, curcumin inhibited VM, reduced cell migration and MMP9 production of the HCC cells. Further study revealed that the anti-VM effect of curcumin was due to inhibition of AKT and STAT3 phosphorylation, as confirmed by specific inhibitors. Conclusion: Curcumin presents proven potential as an anti-VM agent in HCC cells, through down-regulation of STAT3 and AKT signaling pathways.*

It was long believed that only endothelial cells contribute to tumor vascularization, until the process of vasculogenic mimicry (VM) within tumor was described (1). VM is a tumor vascularization that mimics vasculogenesis of endothelial cells, but the blood channels are formed by highly aggressive cancer cells without endothelial cell lining (1). VM has been found in several cancer types, for example, melanoma, ovarian carcinoma, mesothelial and alveolar rhabdomyosarcoma, hepatocellular carcinoma (HCC), colorectal cancer, and laryngeal squamous cell carcinoma (1-6). Importantly, conventional anti-angiogenic agents seem unable to inhibit VM, since three angiogenesis inhibitors (anginex, TNP-470 and endostatin) did not inhibit *in vitro* VM by melanoma cells (7). Moreover, a recent study in an animal model of ovarian cancer revealed that inhibition of angiogenesis by bevacizumab, a vascular endothelial growth factor (VEGF)-neutralizing antibody, promotes VM within the tumor (8). This suggests that VM may be a mechanism of resistance to anti-angiogenic therapy. Since inhibition of VM may improve cancer therapy, the search for anti-VM agents is of great interest.

VM appears to correlate with the rate of metastasis and VM contributes to metastatic progression (4-6). Accumulating data indicate that VM capacity is strongly associated with the invasive phenotype of cancer cells. High cell motility and the ability to re-model the extracellular matrix (ECM) by producing matrix metalloproteinase enzymes (MMPs) are characteristics of highly invasive cancer cells. This is similar to the mesenchymal phenotype which can be acquired in epithelial-derived cancer cells *via* the epithelial–mesenchymal transition (EMT) (9). We previously demonstrated a correlation between VM capacity, invasive phenotype and mesenchymal phenotype in human HCC cell lines, where the VM capacity of a less invasive HCC cell line was enhanced by a pro-metastatic agent such as hepatocyte growth factor. This induced EMT and increased invasiveness of the HCC cells (10). Therefore, we hypothesize that any agent that can suppress invasiveness of cancer cells might abolish VM.

Phosphoinositide 3-kinase (PI3K)/AKT, extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3), and nuclear factor-κB (NF-κB)

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Key Words: Curcumin, vasculogenic mimicry, hepatocellular carcinoma, antimetastatic, natural products.

signaling pathways are usually activated in highly invasive cancer cells. These invasion-modulated signaling pathways have been described as important modulators of VM in different types of cancer. For example, activation of PI3K/AKT and ERK signaling pathways are necessary for VM of melanoma cells (11-12), while the important role of STAT3 in VM was described in ovarian cancer cells (13). It is interesting to explore the role of these signaling pathways in VM of HCC cells.

Curcumin is a natural bioactive product found in turmeric (*Curcuma longa*), shown to exhibit several anticancer properties (14). The inhibitory effect of curcumin on cancer progression is known to result from its interaction with multiple proteins in a variety of biochemical pathways (15). The anti-invasive activity of curcumin has been shown by down-regulation of MMP2 and MMP9 (16), or by decreased cell migration (17). Until now, only one report presents the effect of curcumin on VM, where in a murine choroidal melanoma model, curcumin reduced VM within the engrafted tumor by reducing the expression of proteins in erythropoietin-producing hepatocellular receptor tyrosine kinase class A2 (EphA2)/PI3K/MMP signaling pathway (18). However, the effect of curcumin on VM of HCC cells has not been reported as far as we are aware.

In the present study, we investigated the inhibitory effect of curcumin on VM of human HCC cells, and its correlation with the anti-invasive effect of the compound. The mechanism of action was elucidated by identifying targets of curcumin in invasion-modulated signaling pathways.

Materials and Methods

Chemicals and antibodies. Curcumin was purchased from Fluka-Chemika (Buchs, Switzerland). Curcumin was dissolved in dimethylsulfoxide (DMSO) and kept as stock solution at −20˚C. The final concentration of DMSO was kept below 0.2% (v/v) throughout the study. Cell culture medium and antibiotics were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). 3-[4, 5-dimeylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Primary antibodies against phospho-AKT (Ser473), phospho-STAT3 (Tyr705), phospho-ERK (Thr202/Tyr204), phospho-p65 NF-κB (Ser536) and its total forms and secondary antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). PI3K-specific inhibitor LY294002 (LY) and STAT3-specific inhibitor cucurbitacin-I (CBC-I) were purchased from Calbiochem (San Diego, CA, USA).

Cell culture. The SK-Hep-1 human HCC cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 125 ng/ml amphotericin B. The cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2.

Cell viability assay. Cell viability was determined by the spectrophotometric measurement of mitochondrial dehydrogenase activity using MTT assay as previously described (19). Briefly, cell suspension in culture media was seeded at 1×10^4 cells in 96-well plates (100 μl/well), and incubated at 37˚C in a humidified atmosphere of 5% CO_2. After 24 h, the cells were treated with media containing test compound or vehicle (100 μl) and incubated for another 24 h. The wells were replenished with fresh culture media containing MTT (0.5 mg/ml) for 2 h at 37˚C. Finally, the media were removed and DMSO was added to the wells (100 μl/well), to dissolve the insoluble purple formazan product into a colored solution. Absorbance was measured at 550 nm and subtracted from that of a reference wavelength at 650 nm, using a microplate reader. The number of viable cells was determined from the absorbance. Assays were performed in triplicate wells. Data are expressed as percent viability compared with control.

Matrigel tube formation assay. *In vitro* VM of cancer cells was assessed by Matrigel tube formation assay as previously described (10). In brief, each well of a 96-well plate was pre-coated with 50 μl of Matrigel. After gel solidification, cell suspension in culture media (1.8×10^4 cells/200 μl) containing test compound was seeded into the wells. After 5 h incubation at 37˚C in a CO_2 incubator, photographs were taken using a digital camera attached to a phase-contrast inverted microscope. The total length of tube-like structures formed including branches, was measured using a digital map-meter (20).

Cell migration assay. Migration of cancer cells was assessed by Transwell chambers (19). Cell suspension in culture media was incubated with test compound or vehicle for 30 min at 37˚C, after which the cell suspension (5×10^4 cells in 200 μl) was added into the upper chamber of the Transwell. The lower chamber was filled with 500 μl of culture media containing the test compound. After 5 h of incubation at 37˚C, the cells on upper surface of filter were swabbed, and the cells attached on lower surface of the filter were fixed with 25% methanol, stained with a solution of 0.5% crystal violet in 25% methanol, and extracted with 0.1 N HCl in methanol. Absorbance was measured at 550 nm and used to determine the number of migrated cells. Data are expressed as the percentage migration compared with that of the control.

Gelatin zymography. Production of MMP9 by SK-Hep-1 cells was determined by gelatin zymography as previously described (10). Sub-confluent culture of cancer cells grown in 24-well plate was rinsed and incubated with serum-free media containing test compound or vehicle (400 μl/well) for 24 h, after that, conditioned media were collected. The conditioned media were resolved in 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/ml gelatin, under non-reducing condition. The gel was washed twice with 2.5% triton X-100 for 30 min to remove SDS, and subsequently incubated for 18 h at 37˚C in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% NaN_3. The gel was stained with 0.3% coomassie blue for 1 h, then de-stained in 10% acetic acid and 30% methanol. Gelatinolytic activity of MMP9 was visualized as clear zones on a blue background.

Immunoblot analysis. Expression of phosphorylated forms and all forms of selected signaling proteins was determined by immunoblot analysis as previously described (21). Cell suspension containing the test compound was seeded into 6-well plate and incubated at 37˚C for 5 h. The cells were rinsed with cold phosphate buffered

saline, then scraped and lysed in radio-immunoprecipitation assay buffer containing a protease inhibitors cocktail (Sigma) and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, and 20 mM β-glycerophosphate). The protein concentration of the cell lysate was determined using Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates (25 μg protein) were subjected to electrophoresis in 7.5% SDS-PAGE, and electrophoretically-transferred to Immobilon-P nylon membrane (Millipore, Bedford, MA, USA). The membrane was probed with indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody. Bands were visualized using ECL reagents (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Statistical analysis. Statistical significance of differences between control and treatment groups was determined by one-way analysis of variance (ANOVA) using statistical software SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA) and a *p*-value less than 0.05 was considered to be significant.

Results

Inhibition of VM by curcumin. Tube-like structures in SK-Hep-1 cells were rapidly formed and observed within 5 h after seeding on Matrigel (Figure 1A). Within the range of 3-30 μM, curcumin inhibited tube formation of the cells in a dose-dependent manner, with 18-92% inhibition being observed (Figure 1A and B). This inhibitory effect of curcumin was not due to its cytotoxicity because more than 90% cell viability was retained throughout the range of test concentrations (Figure 1B). The results indicate the anti-VM potential of curcumin in human HCC cells.

Curcumin inhibited cell migration and MMP9 production. We previously showed that the VM capacity of HCC cells was strongly associated with an invasive phenotype, which is determined by cell migration capability and MMP production (10). Therefore, we further investigated whether the anti-VM activity of curcumin is associated with suppression of the invasive phenotype of the cells. When the effect of curcumin on migration of SK-Hep-1 cells was tested, a significant inhibition was observed at a concentration of 30 μM curcumin (Figure 2A). Additionally, gelatin zymographic analysis revealed that curcumin treatment suppressed MMP9 production of SK-Hep-1 cells, as clearly seen at a concentration of 30 μM curcumin (Figure 2B). The inhibitory effects of curcumin on cell migration and MMP9 production concur with its inhibition of tube formation, suggesting that the anti-VM activity of curcumin resulted from reduction of cancer cell invasiveness.

Effects of curcumin on multiple signaling pathways. We further explored the effect of curcumin on invasion-modulated signaling pathways that may be involved in VM of HCC cells, including PI3K/AKT, STAT3, ERK, and NF-κB. Constitutive activation of these signaling pathways was found in SK-Hep-1 cells (Figure 3), where phosphorylation of AKT is an intracellular indicator of PI3K activity. Curcumin treatment (30 μM) for 5 h reduced constitutive phosphorylation of AKT and STAT3 in the cells, while phosphorylation of ERK and p65 NF-κB were not affected (Figure 3). The concurrent inhibition of AKT and STAT3 phosphorylation, and suppression of invasive phenotype and VM capacity of the cells by curcumin treatment suggests AKT and STAT3 might be targets of curcumin in this cell line.

Inhibition of AKT and STAT3 phosphorylation suppressed VM. In order to confirm that the anti-VM effect of curcumin was mediated through the inhibition of AKT and STAT3 phosphorylation, specific inhibitors of PI3K/AKT (LY, 25 μM) and STAT3 (CBC-I, 10 μM) were used to treat SK-Hep-1 cells. The inhibitors reduced phosphorylation of AKT and STAT3, similarly to what was observed with 30 μM curcumin treatment (Figure 4A). VM of SK-Hep-1 cells was significantly reduced by both inhibitors, with 60% inhibition by LY and 90% inhibition by CBC-I (Figure 4B). We further determined the effect of the specific inhibitors on the invasive phenotype of the cells. LY and CBC-I had differential effects on migration of SK-Hep-1 cells. At the concentration that inhibited tube formation, CBC-I significantly reduced cell migration by 75% inhibition, while LY did not (Figure 4C). However, both LY and CBC-I similarly suppressed MMP9 production of SK-Hep-1 cells (Figure 4D), suggesting that MMP9 production in this cell line was regulated by PI3K/AKT and STAT3 signaling pathways, and confirming the contribution of MMP9 to VM of SK-Hep-1 cells. Collectively, our results demonstrate that curcumin inhibited VM of human HCC cells, suppressing their invasive phenotype through inhibiting constitutive activation of AKT and STAT3 in the cancer cells.

Discussion

VM is currently considered a mechanism of cancer resistance towards anti-angiogenic drugs. It is not clear how cancer cells form channels by VM within the tumor. In the Matrigel tube formation assay, both endothelial cells and highly invasive cancer cells can form tubular structures on the surface of Matrigel. During tube formation, endothelial cells re-model the ECM using MMPs, and migrate to organize into tubular structures (22), with most steps of this process being similar to cancer invasion. VM of highly invasive cancer cells would be expected to be similar to endothelial tube formation. Thus, an anti-invasive agent that suppresses migration or MMP production of cancer cells might inhibit VM. Our hypothesis was supported by evidence from other studies using the siRNA approach to reduce cancer invasiveness by silencing expression of EMT regulators, Twist family bHLH transcription factor-1 (Twist1) or Zinc

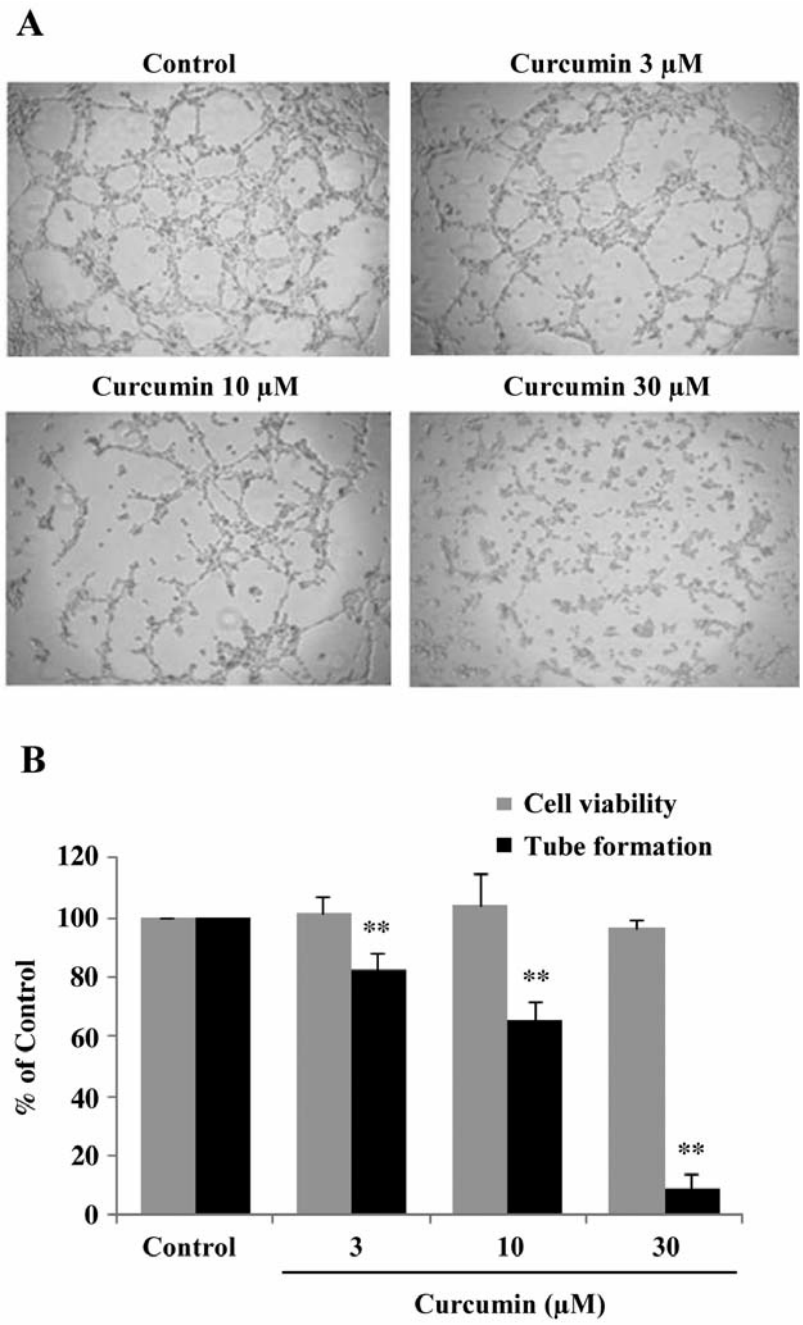

Figure 1. *Effect of curcumin on vasculogenic mimicry (A) and viability (B) of SK-Hep-1 cells. A: Phase-contrast images of tube formation in the presence of curcumin. The cells were seeded into Matrigel-coated wells and allowed to form tubular structures in the presence or absence of curcumin for 5 h; original magnification of ×100. B: Cell viability was determined after incubating cells with different concentrations of curcumin for 24 h. Total tube length of the tubular network was quantified from the photographs taken 5 h after seeding. Data are expressed as the mean±SD of three independent experiments, and significant differences from the control are indicated by **p<0.01.*

finger E-box binding homeobox 1 (ZEB1), resulting in suppression of the VM capacity of HCC and colorectal cancer cells (5, 23).

As far as we are aware of, the present study is the first to report the anti-VM potential of curcumin in an HCC model by inhibiting *in vitro* VM of SK-Hep-1 HCC cells. The anti-VM effect of curcumin results from inhibiting migration and MMP9 production of the cells. Curcumin is known to have multiple targets, and our results clearly showed that inhibition of PI3K/AKT and STAT3 signaling pathways were

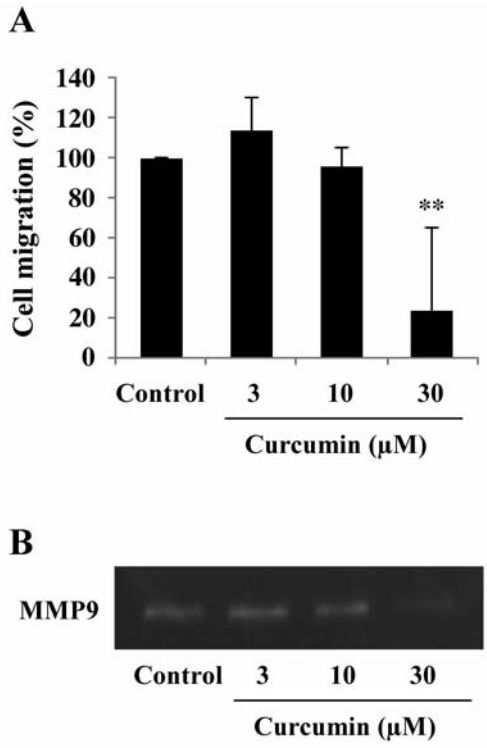

Figure 2. *Effect of curcumin on the invasive phenotype by cell migration (A) and MMP9 production (B) in SK-Hep-1 cells. A: The cells were allowed to migrate in Transwell chambers for 5 h in the presence or absence of curcumin. Data are expressed as the mean±SD of three independent experiments, and significant differences from the control are indicated by **p<0.01. B: Cells were treated with curcumin in serum-free media for 24 h, and then conditioned media were collected and analyzed by gelatin zymography. MMP9 activity in conditioned media was revealed as clear bands on a blue background.*

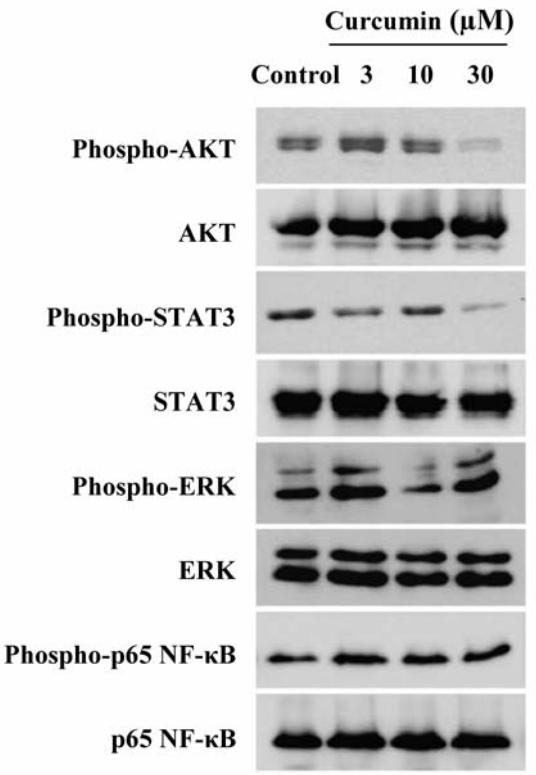

Figure 3. *Effect of curcumin on invasion-modulated signaling pathways involved in the regulation of cell migration and MMP9 production. SK-Hep-1 cells were treated with curcumin for 5 h. Whole-cell lysates were subjected to immunoblot analysis for determining levels of total and phosphorylated forms of AKT, STAT3, ERK and NF-κB p65 proteins.*

a mechanism underlying the anti-VM effect of curcumin. The important roles of PI3K/AKT and STAT3 signaling pathways in VM of HCC cells were confirmed by treatment with specific inhibitors (Figure 4B). PI3K has been shown to play a role in VM of melanoma cells through regulation of ECM re-modeling, by promoting conversion of pro-MMP2 into the active form, resulting in increased ability of melanoma cells to re-model ECM and subsequently form VM (11). Although MMP2 was not detected in SK-Hep-1 cells, we previously showed that this VM-positive HCC cell line produced a high level of MMP9 compared with VM-negative HCC cell line, HepG2 (10). MMP2 and MMP9 belong to the same sub-family, sharing substrate specificity. This may have partially functional redundancy because impairment of invasion and tumor vascularization were observed only in doubly-deficient mice lacking MMP2/MMP9, but not in those with single MMP deficiency (24). The contribution of PI3K/AKT signaling in MMP9 production of cancer cells has been reported (25). PI3K/AKT-specific inhibitor treatment reduced VM, as well as the production of MMP9 of SK-Hep-1 cells, without significant effect on cell migration (Figure 4C and D). Our results suggest that PI3K/AKT signaling plays a role in VM of HCC cells through regulation of ECM remodeling, similar to that observed in melanoma formation of vessels by VM.

Additionally, our results also reveal involvement of STAT3 in VM of SK-Hep-1 cells, by regulating both MMP9 production and cell migration (Figure 4). These results agree with a recent report showing an inhibitory effect of STAT3 inhibitor in VM of ovarian cancer cells (13). STAT3 plays several roles in the promotion of cancer metastasis, and we showed that inhibition of STAT3 by a flavonoid chrysin suppressed growth of metastatic colonies of 4T1 murine breast cancer cells *in vivo* (21). A correlation between MMP9 expression and persistently tyrosine-phosphorylated STAT3 was shown in breast cancer tissues; the MMP9 level is up-regulated by STAT3 at the transcriptional level during transformation of mammary epithelial cells (26). STAT3 is

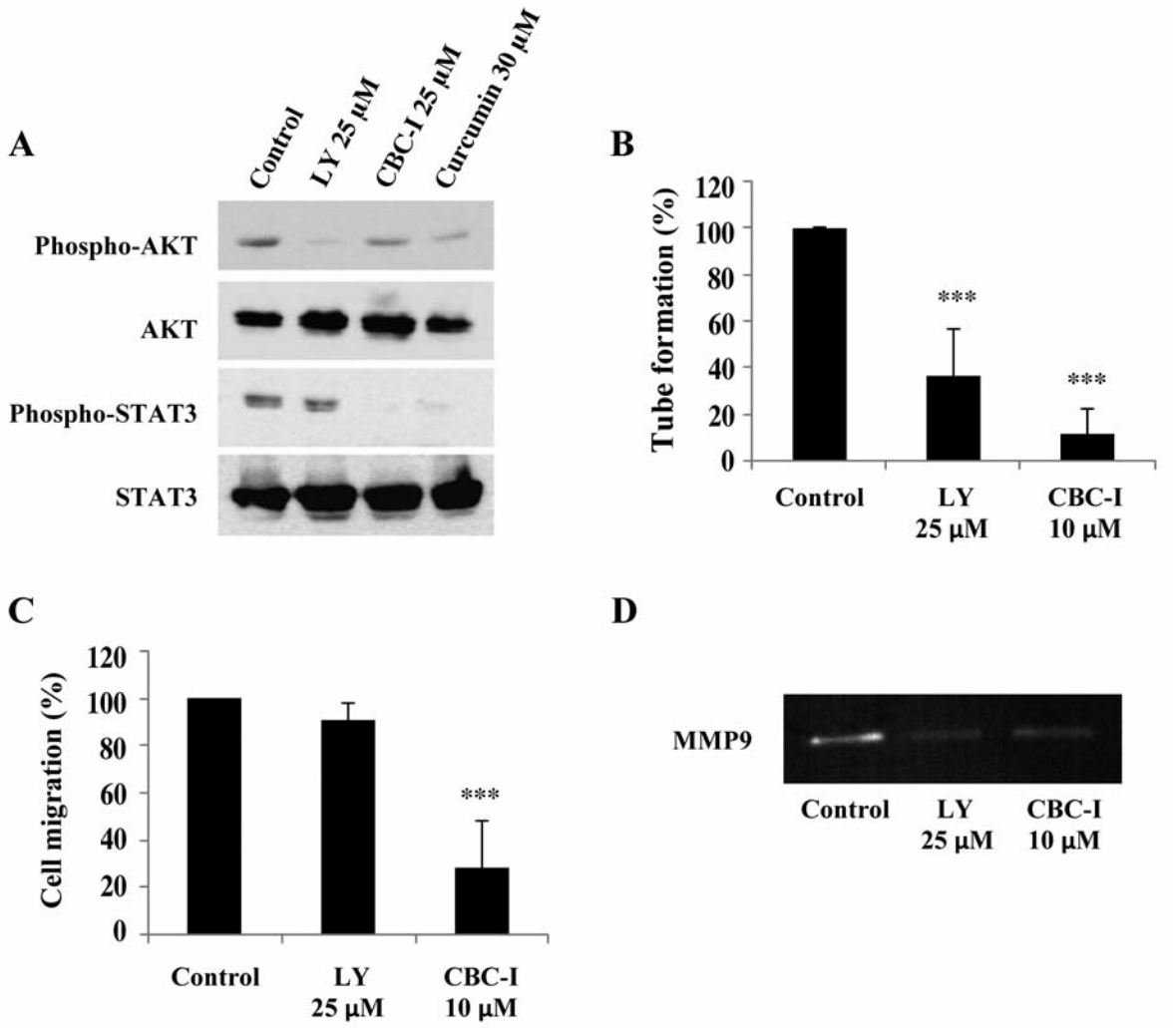

Figure 4. *Effect of specific inhibitors of PI3K/AKT (LY, 25 μM) and STAT3 (CBC-I, 10 μM) signaling pathways on invasive phenotype and VM of SK-Hep-1 cells. A: Cells were treated with test compounds for 5 h, then whole-cell lysates were subjected to immunoblot analysis for determining levels of total and phosphorylated forms of AKT and STAT3 proteins. B: Cells were seeded into Matrigel-coated wells and allowed to form tubular structures in the presence of test compounds for 5 h, then phase-contrast images were taken and total tube length of the tubular network was quantified from the photographs. C: SK-Hep-1 cells were allowed to migrate in Transwell chambers for 5 h in the presence of test compounds for 5 h. D: Cells were treated with test compounds in serum-free media for 24 h, then conditioned media were collected and analyzed by gelatin zymography. MMP9 activity in conditioned media was revealed as clear bands on a blue background. The data are expressed as mean±SD of three independent experiments, and significant differences from the control are indicated by ***p<0.001.*

also involved in the cell migration process through transcription-dependent and -independent functions (27). Thus, inhibiting STAT3 functions in cancer cells may be a promising strategy for preventing metastasis. The multiple targets of curcumin in VM-related signaling pathways is an advantage over single target-specific inhibitors, since more than one signaling pathway may contribute to VM of cancer cells, and they may operate with functional redundancy.

Although there is increasing research on VM, only few agents have been shown to possess anti-VM potential. For example, thalidomide suppresses VM of melanoma by reducing expression of NF-κB, VEGF, MMP2 and MMP9, and proliferating cell nuclear antigen (PCNA) (28). Genistein reduces VM of melanoma by down-regulating vascular endothelial (VE)-cadherin (29). Isoxanthohumol also reduces VM of breast cancer cells (30).

In summary, we have shown that curcumin exhibited an anti-VM effect through suppressing the invasive phenotype of cancer cells. We suggest that instead of using an angiogenesis inhibitor, anti-invasive or anti-metastatic agents inhibiting migration or MMP production may be of potential use in anti-VM therapy, as well as in prevention of metastasis.

Acknowledgements

This study was supported by a research grant from the Chulabhorn Research Institute. We would like to thank Ms. Korakot Atjanasuppat for technical assistance.

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Received January 16, 2014
Revised February 15, 2014
Accepted February 17, 2014