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Full Length Research Paper

Curcumin inhibits cell survival and migration by suppression of Notch-1 activity in prostate cancer cells

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Previous studies have indicated that Notch-1 activity plays an essential role in prostate tumorigenesis. However, its underlying mechanism is not yet clear. In this study, Curcumin, a drug widely used in Ayurvedic medicine for its antimalarial and anti-inflammatory properties, was used to investigate its impact on cell survival, migration and Notch signaling status in prostate cancer cell lines. Our data show that Curcumin treatment significantly suppressed the proliferation and migration of prostate cancer cell lines DU145 and PC3 in a dose- and time-dependent manner. Two migration-related genes, MT1-MMP and its target-molecule MMP2, were downregulated by Curcumin. There was no significant change of Notch-1 and its cleaved product NICD levels after Curcumin treatment. Furthermore, our chromatin immunoprecipitation assay (ChIP) revealed a remarkable decrease of NICD binding to Hes-1 promoter. Altogether, our results suggest an anti-tumor action of Curcumin in prostate cancer that might be through suppression of the Notch-1 transactivity.

Key words: Prostate cancer, Curcumin, Notch.

INTRODUCTION

Prostate cancer remains the second leading cause of cancer-related death among men in the world (Carlsson et al., 2012). The high mortality of this life-threatening disease results from a bundle of factors, such as late diagnosis, high metastatic potential and lack of effective therapies available, etc. These disappointing facts call for cancer researchers to pay much more attention to prostate cancer. Recent molecular and cellular *in vivo* and *in vitro* studies have indicated that the activation and deactivating of multiple cellular signaling pathways might be involved in the development and progression of prostate cancer (Mellado et al., 2009; Sarker et al., 2009; Yang and Dou, 2010). Among all the possible pathways that might be associated with prostate cancer development, Notch pathway is increasingly gaining attention in recent years. Notch signaling pathway is an evolutionarily conserved signaling system essential for embryonic development in metazoan (Dang, 2012). In eukaryotic cells, Notch family consists of four Notch receptors (Notch14), three Deltalike ligands (Dll1, Dll3, and Dll4), and two ligands of the Jagged family (Jag1 and Jag2) (Allenspach et al., 2002; Wang et al., 2009). Notch signaling is initiated by the interaction of its ligands and receptors, resulting in subsequent proteolytic digestion of Notch receptor by two enzymes. The first cleavage is conducted by an extracellular matrix metalloprotease, followed by second cleavage mediated by the transmembrane protease complex γ-secretase, releasing

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the Notch intracellular domain (NICD) (Miele et al., 2006). The released NICD can translocate into the nucleus where it, interacting with the CSL family of transcription factors (CBF-1/RBP-Jk, Su (h) and LAG-1), regulates its target genes, such as Hes-1and Hey-1, which are involved in cell survival regulation. Several lines of evidence showed that suppression of Notch-1 activation contributed to cancer cell growth inhibition and apoptosis onset. More recent data indicated that Notch-1 signal might regulate prostate cell proliferation by targeting Hes-1(Beatus et al., 2001; Zhang et al., 2009).

Curcumin, a yellow pigment from *Curcuma longa*, is a widely-used spice in Southeast Asian and Middle Eastern cuisine (Ravindran et al., 2009). Several biochemical and functional studies have indicated that Curcumin possesses a potent anti-cancer activity in many types of cancer (Shishodia et al., 2007), especially in prostate cancer (Kurien and Scofield, 2009; Teiten et al., 2010). Since Notch signaling pathway is one of the most essential pathways implicated in prostate cancer development, in this study, our aim is to elucidate if Notch signalling is involved in Curcumin's anti-cancer activity in prostate cancer cell.

MATERIALS AND METHODS

Reagents and kits

All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). BCA protein quantitation kit was purchased from Pierce Company, USA. SYBR® Premix Ex Taq™ II (Perfect Real Time) was purchased from Takara (Dalian, China). Curcumin, purchased from Calbiochem (La Jolla, CA), was dissolved in DMSO and stored at -20°C.

Cell culture

The DU145 and PC3 cells, two prostate cancer cell lines, were purchased from American Type Culture Collection (Rockville, MD, USA) and were cultured in complete medium (RPMI-1640 medium supplemented with 10% FBS, streptomycin 100 mg/ml and penicillin 100 U/ml) at 37°C in 5% CO_2 humidified incubator.

MTT assay

Cells were seeded with 100 µl of complete medium in 96-well plate (5×10^3 cells per well). Different doses of Curcumin were added to each well for indicated times. After 48 h of Curcumin treatment, MTT reagent (5 mg/ml) was added to each well, and incubated for 4 h at 37°C. The formazan crystals were solubilized by the addition of 100 µl of DMSO. The optical density (OD) at 570 nm was measured and cell viability was determined by the following formula. Cell viability (%) = (OD of the treated wells - OD of the blank control wells) / (OD of the negative control wells - OD of the blank control wells) ×100%. All MTT experiments were performed in triplicate and repeated at least three times.

Wound healing assay

Cells were plated into 6-well plates and grown to full confluence. The cell monolayer was artificially wounded using a 200-µl pipette tip. Cell debris was removed by washing with PBS. After treatment of Curcumin for indicated times, wound closure was photographed with an inverted microscopy equipped with a digital camera. The wound healing extent was defined as the ratio of the difference between the original and the remaining wound areas compared with the original wound area. All experiments were performed in the presence of 10 µg/ml of mitomycin-C, a cell proliferation inhibitor.

Western blot

The Curcumin-treated and untreated cells were lysed in modified RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 mM EDTA, 100 mM NaF, and 1 mM Na_3VO_4) containing protease inhibitor cocktail. Cell lysates were spun at 12000 rpm for 30 min to collect supernatant. Protein concentration was determined by the BCA kit. Total proteins were separated by SDS-PAGE and transferred to PVDF membrane. After blockage in 5% nonfat milk, blots were incubated overnight at 4°C with primary antibodies. After washing by PBST (PBS-Tween), the blots were incubated for 1 h with horseradish peroxidase (HRP) conjugated secondary antibody. After washing in PBST, blots were visualized using enhanced chemiluminescence (ECL, Pierce, USA), followed by exposure to Fujifilm LAS3000 Imager (Fuji, Japan). The band densities on the blots were normalized relative to the relevant β-actin band density with Quantity One software (Bio-Rad, USA).

ChIP assay

After treatment of Curcumin for the indicated times, cells were fixed with 1% formaldehyde and nuclei were isolated. After sonication, soluble chromatin DNA was precleared with protein A beads slurry and salmon sperm DNA. Precleared chromatin was immuno-precipitated overnight with 2 µg of antibody for Notch-1 and normal rabbit IgG which serves as negative control. After extensive washing, de-crosslinking and purification, routine PCR was performed to detect the target Hes-1 promoter by using the following primer pair: Forward, 5'-CTGAAAGTTACTGTGGG-3'; reverse, 5'-TGAGCAAGTGCTGAGGG-3'. β-actin (forward 5'-CTGGAACGGTGAAGGTGACA -3'; reverse, 5'-AAGGGACTTCCTGTAACAATGCA-3') was used as a loading control. qPCR was also performed to make quantitative analysis. The ChIP qPCR signals of Curcumin treatment are subtracted by the IgG signals. The IgG-normalized ChIP data was presented as percentage of control (0h), which was arbitrarily set as 100%.

Statistical analysis

Results in this study were expressed as mean ± standard deviation (SD). Student's t-test was used for statistical analysis with SPSS 16.0. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Curcumin decreased cell viability in prostate cancer cell lines

First, we determined the influence of Curcumin treatment on cell survival. DU145 and PC3 cells were treated with increasing concentration of Curcumin for the indicated time, and MTT assay was performed to examine cell

viability. As shown in Figure 1A, the viability of both DU145 and PC3 cells was significantly inhibited in a dose-dependent manner. Significantly inhibitory effect was noted between the doses of 10 μM and 50 μM ($p<0.01$). Also, we observed cell viability after treatment of 25 μM of Curcumin for increasing hours. The result was shown in Figure 1B. Obviously, Curcumin decreased cell viability in a time-dependent manner. After 48 h Curcumin treatment, both PC3 and DU145 cells experienced a maximal cell viability loss (~50%) ($P<0.05$).

The migration of prostate cancer cell lines was inhibited by Curcumin treatment

Next, we assessed the impact of Curcumin treatment on cell migration. We performed a classical cell wound healing assay to determine the cell migration ability. To exclude the impact of cell proliferation, mitomycin-C was included in this assay as described in materials and methods. As shown in Figure 2, Curcumin dramatically suppressed DU145 cell mobility compared to control treatment, with a statistically significant difference ($P<0.05$).

Curcumin downregulated the expression of MT1-MMP and MMP2, but not of NICD

To test whether Curcumin could affect the expression of Notch-1 and its cleaved form, Notch-1 intercellular domain (NICD), we used western blotting to detect their levels in DU145 cells upon Curcumin treatment. Our study revealed that different concentrations of Curcumin (10 or 50 μM) resulted in no obvious changes of Notch-1 and NICD levels (Figure 3). Since the above results (Figure 2) showed a migration-inhibitory effect of Curcumin, we also determined two migration-related genes expression levels. As shown in Figure 3, MT1-MMP and MMP2 levels were both decreased after Curcumin treatment (Figure 3).These results suggested that Curcumin could down-regulate the expression of MT1-MMP and MMP2 without affecting Notch-1 and NICD levels.

Curcumin suppressed the binding of NICD to Hes-1 promoter

The above results indicated that the downregulation of Notch pathway target genes induced by Curcumin might be duo to the changes of the quality, but not of the quantity of Notch-1. So, we further examined the transactivating ability of NICD upon Curcumin treatment. To this end, chromatin immunoprecipitation assay was conducted to detect the NICD binding to the promoter of Hes-1, one of its target genes. As shown in Figure 4A, in control treated cells, we using PCR obtained a strong amplicon. In sharp contrast, the amplicon from Curcumin-treated cells showed a fainter amplifying signal, indicating a decreased binding activity of NICD. To better show the changes in NICD binding to Hes-1 promoter, we also performed qPCR. After 10 or 50 μM Curcumin treatment, NCID binding to Hes-1 promoter was decreased to 53.8 and 14.5% respectively (Figure 4B).

DISCUSSION

Curcumin has gained much attention in cancer research field during the last decade. Curcumin has been demonstrated to inhibit almost many types of cancer cell, such as head and neck carcinoma (Wilken et al., 2011), colon cancer (Patel et al., 2010) and leukemia (Kelkel et al., 2010). Recently, some researchers reported an anti-neoplastic activity of Curcumin in prostate cancer (Hilchie et al., 2010; Piantino et al., 2009; Teiten et al., 2010). Hilchie et al. (2010) found that Curcumin treatment caused a significant PC3 cell death by inducing apoptosis in a dose and time dependent manner. In the present study, we also found that Curcumin can decrease cell survival of PC3 and another prostate cancer cell line DU145. This effect also depended upon dose and duration of treatment. Another study using DU145 as experiment model also obtained a similar conclusion that Curcumin can strongly suppress cell survival (Mukhopadhyay et al., 2001). Therefore, in combination with all these previous findings, our results support an anti-cancer bioactivity of Curcumin in prostate cancer.

In this study, Curcumin suppressed not only cell survival but also cell migration. As we know, increased cell migration is another characteristic of tumor cells. Therefore, curbing tumor cell migration is regarded as a tumor therapeutic target. A recent biochemical study showed that Curcumin bears a strong migration-suppressing activity in lung cancer cells (Yang et al., 2012). Another study using microglial cells as model also gained a parallel conclusion that Curcumin is a negative cell migration regulator in tumor cells (Karlstetter et al., 2011). These findings agree well with our results in prostate cancer cells. Furthermore, we also attempted to interpret its underlying mechanism and found MT1-MMP and MMP2 might be the molecular target of Curcumin, because these two important migration-relating genes can be downregulated by Curcumin.

To date, multiple signaling pathways have been proposed to be implicated in the negative regulation of cancer cell by Curcumin. Indeed, a diverse range of factors have been verified to a molecular target of Curcumin. Curcumin can activate caspases to induce cell apoptosis (Park and Lee, 2007; Tan et al., 2006). Curcumin also can induced cell apoptosis by suppressing the expression of negative apoptosis regulators, such as Bcl-2, Bcl-xL, surviving, or by upregulating protein levels

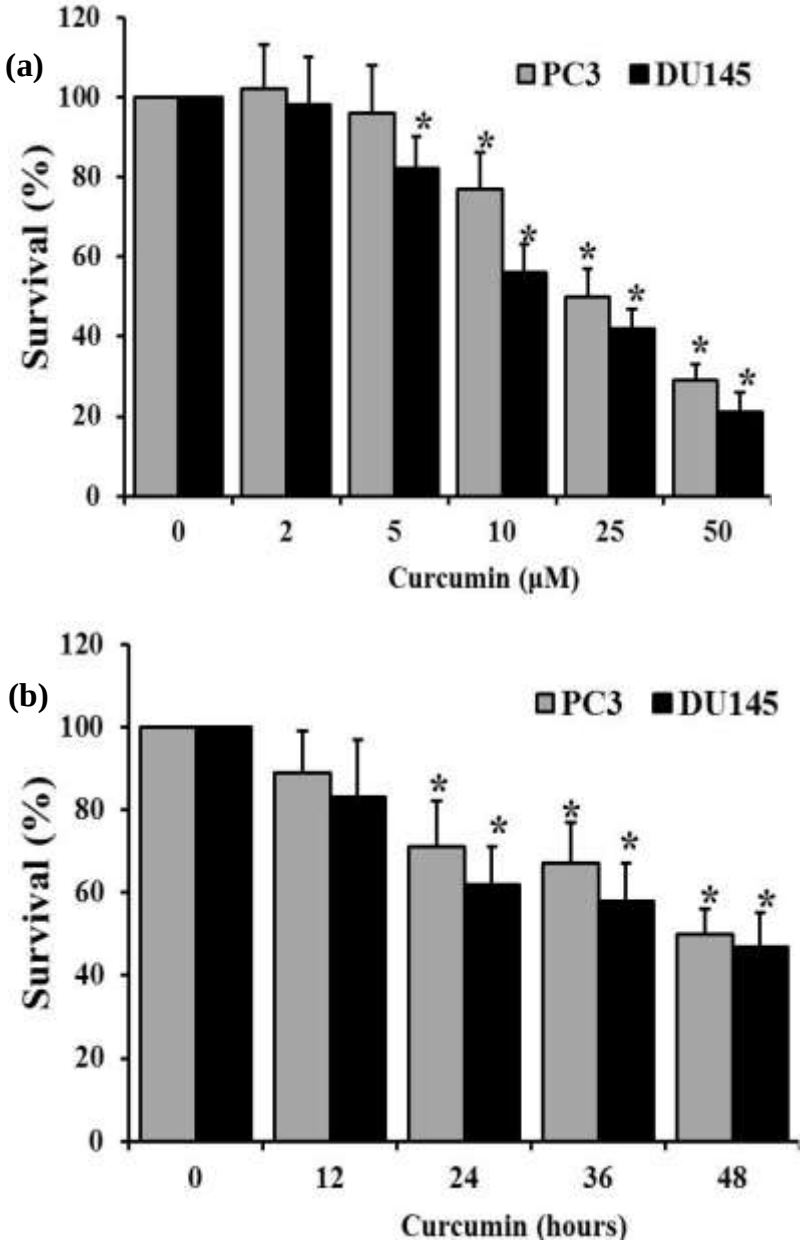

Figure 1. The impact of curcumin treatment on cell survival. **(A)** PC3 and DU145 cells were treated with increasing doses of curcumin for 48 h and MTT assay was performed to examine cell viability. Data from three independent experiments were statistically analyzed and plotted as Mean ± SD. * denotes $P < 0.05$. **(B)** PC3 and DU145 cells were treated with 25 μM of curcumin for increasing hours and MTT assay was performed to examine cell viability. Data from three independent experiments were statistically analyzed and plotted as mean ± SD. * denotes $P < 0.05$.

of positive apoptosis regulators, such as Bax, Bim, PUMA (Ravindran et al., 2009). Curcumin also can induce tumor cell apoptosis in a p53/p21-denpendent manner (Liu et al., 2007; Srivastava et al., 2007). Here, our results indicated another important cellular pathway, Notch pathway, might be involved in Curcumin's anti-proliferative process, because NICD's transactivating activity was dramatically suppressed by Curcumin. Of note, Curcumin cannot disturb NICD level or total Notch-1 level, suggesting Curcumin can only affect Notch pathway activity, but not its constitution. Altogether, the present study demonstrates a suppressing role of Curcumin in prostate cancer cells. It can inhibit cell proliferation and migration. The involving mechanism might be duo to

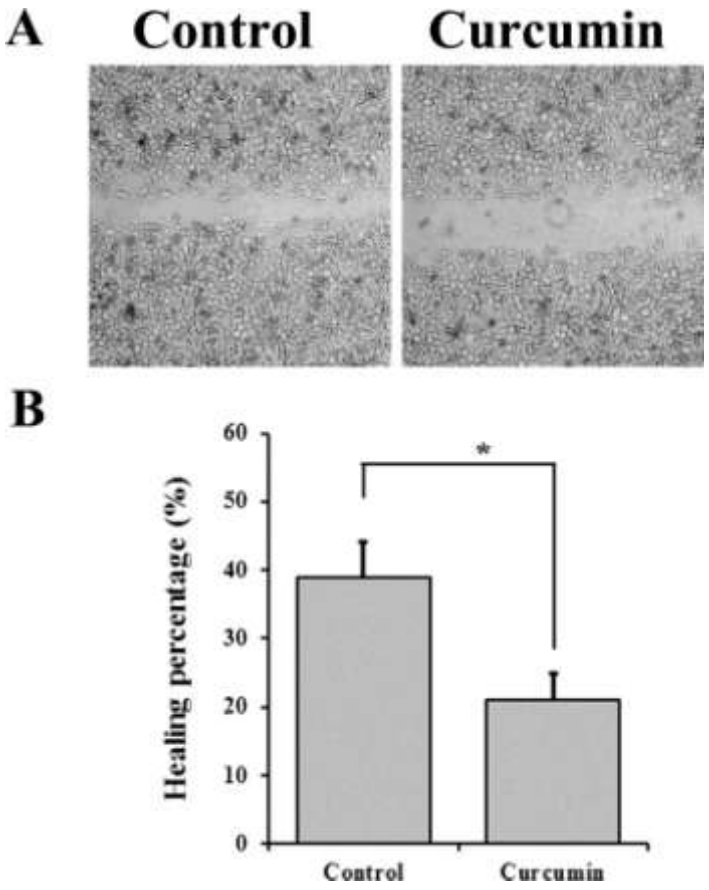

Figure 2. The effect of curcumin treatment on cell migration. Cell wound healing assay was performed to assess the DU145cell mobility after treatment of 25 μM of curcumin or DMSO (as control) for 24 h.

Data from three independent experiments were statistically analyzed and plotted as Mean ± SD. * denotes $P < 0.05$.

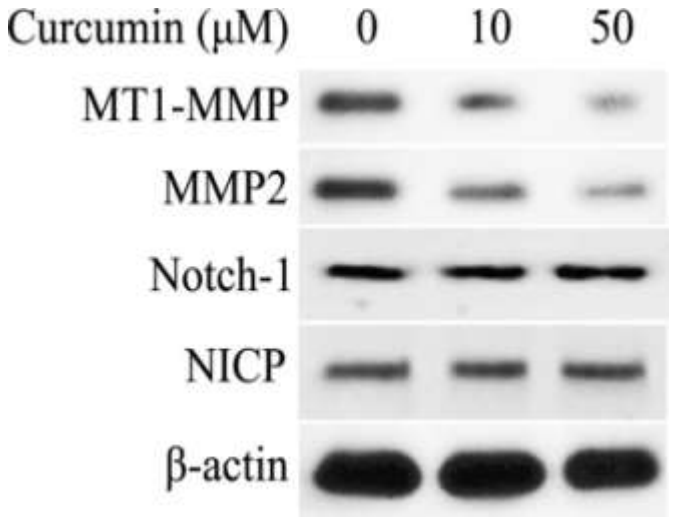

Figure 3. The protein levels of MT1-MMP, MMP2, Notch-1 and NICD after Curcumin treatment. DU145 cells were incubated with 10 μM or 50 μM of Curcumin for 24 h and western blot was performed to detect the protein levels of MT1-MMP, MMP2, Notch-1 and NICD. β-actin served as a loading control.

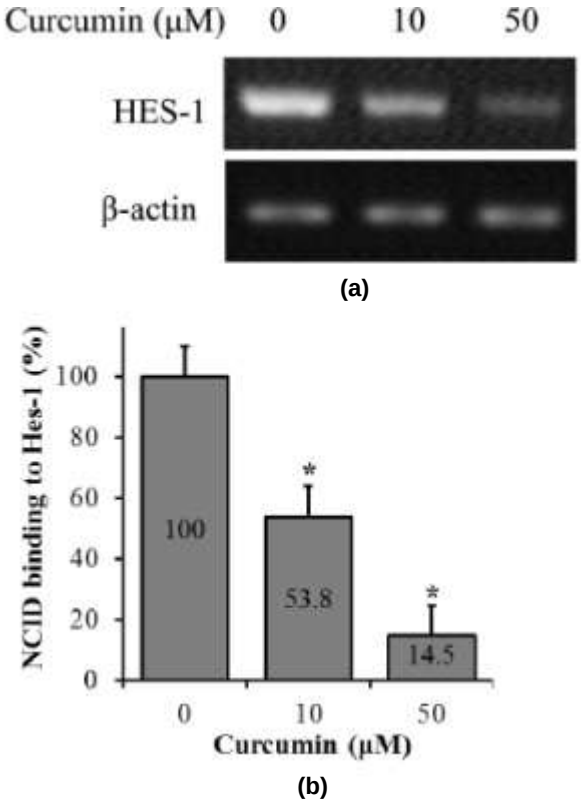

Figure 4. The transactivating ability of NICD after curcumin treatment. DU145 cells were treated with 10 μM or 50 μM of curcumin for 24 h and chromatin immunoprecipitation assay was performed to detect the binding of NICD to Hes-1 gene promoter.

(A) PCR was conducted to detect the Hes-1 promoter segments immunoprecipitated by NICD. (B) qPCR was performed to quantitatively determine the abundance of Hes-1 promoter segments immunoprecipitated by NICD, which represents the NCID binding activity to Hes-1. Data from three independent experiments was statistically analyzed and plotted as Mean ± SD. * denotes $P < 0.05$.

activity loss of Notch signalling. Future study should emphasize the more detailed molecular mechanism underlying this phenomenon.

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