Curcumin inhibits the survival and metastasis of prostate cancer cells via the Notch-1 signaling pathway

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Prostate cancer is one of the most common malignancies in men, and it urgently demands precise interventions that target the signaling pathways implicated in its initiation, progression, and metastasis. The Notch-1 signaling pathway is closely associated with the pathophysiology of prostate cancer. This study investigated the antitumor effects and mechanisms of curcumin, which is a well-known natural compound from curcuminoids, in prostate cancer cells. Viability, proliferation, and migration were analyzed in two prostate cancer cell lines, DU145 and PC3, after curcumin treatment. Whether the Notch-1 signaling pathway is involved in the antitumor effects of curcumin was examined. Curcumin inhibited the survival and proliferation of PC3 and DU145 cells in a dose- and time-dependent manner and inhibited DU145 migration. Curcumin did not affect the expression of Notch-1 or its active product NICD, but it did inhibit the expression of MT1-MMP and MMP2 proteins in DU145 cells. We found that curcumin inhibited the DNA-binding ability of NICD in DU145 cells. In conclusion, curcumin inhibited the survival and metastasis of prostate cancer cells via the Notch-1 signaling pathway.

Key words: Curcumin; prostate cancer; Notch signaling pathway.

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Prostate cancer is one of the most commonly diagnosed malignancies in men. Numerous studies demonstrated that an imbalance in the expression of many genes is associated with prostate oncogenesis. A growing number of genes, including RNAseL, PCAP, hPCX, CAPB, hSD3B, MSR1, NBS1, ChEK2, and others, are implicated in hereditary prostate cancer (1). However, most prostate cancers are sporadic without familial or hereditary features. Many studies of sporadic cancer indicated that oncogenes and anti-oncogenes play pivotal roles in the pathogenesis. For example, the tumor protein p53, commonly known as ‘the guardian of the genome’, is involved in prostate oncogenesis. PTEN and GST are also repeatedly involved in the development of prostate cancer. Recent decades revealed that multiple intracellular oncogenes and anti-oncogenes are implicated in the initiation, progression, and metastasis of prostate cancer. These genes provide good targets for prostate cancer treatment in the future.

Numerous studies demonstrated that many molecules in the Notch signaling pathway are expressed in prostate cancer cells (2). The Notch signaling pathway is a highly conserved signaling system in evolution, and it exhibits diversified functions, including cell proliferation, differentiation, and apoptosis (3). The Notch signaling pathway plays a critical role in regulating cell fate determination during the development of multicellular organisms. There are four Notch receptor genes in humans (4). The Notch receptor possesses an extracellular domain that is composed of multiple epidermal growth factor-like (EGF) repeats, a single transmembrane pass, and an intracellular domain with a nuclear localization signal (Notch intracellular domain, NICD). The NICD is the activated form of Notch (5), and its activation requires three proteolytic cleavages (4). The activated NICD moves into the nucleus, binds with the transcription factor RBPJ/CSL (‘CBF-1, Suppressor of Hairless, Lag-2’,
after its mammalian, Drosophila, and Caenorhabditis elegans orthologs, and regulates the transcription of many target genes, including Hes, Hey, HERP, bHLH, cyclin D1, cyclin A, and NF-xB (6). Notch ligands primarily consist of Jagged1, Jagged2, and Delta-like 1, 3, and 4 (7).

Notch and NICD are expressed in four commonly used cell lines of prostate cancer, and cell proliferation and invasion are inhibited by blocking Notch activation (8). Clinicopathological data revealed that most members in the Notch signaling pathway are highly expressed in prostate cancer. Hafeez et al. (2) found that the expression level of Notch-1 is remarkably associated with Gleason scoring for prostate cancer. A poor score corresponds to high Notch-1 expression in cancer tissue, and a better score indicates lower expression, which suggests a positive correlation between Notch-1 expression and prostate cancer malignancy. Cells with high Notch-1 expression occur primarily around blood vessels, which suggests that these cells may more easily penetrate the blood vessels and enter the circulation to initiate metastasis. Cells from metastasized prostate cancer are primarily accompanied with increased Jagged1 expression (9). Jagged1 and Notch-1 colocalize in prostate cancer cells, and this provides further evidence that Notch-1 signaling activity is closely linked with the metastatic potential of prostate cancer.

Curcumin is a yellow pigment from Curcuma longa, which is a widely used spice in Southeast Asian and Middle Eastern cuisines. Several biochemical and functional studies indicated that curcumin possesses a potent anti-cancer activity against many types of cancer, especially prostate cancer. The Notch signaling pathway is an essential pathway in prostate cancer development. Our study elucidated the role of Notch signaling in the antitumor activity of curcumin in prostate cancer cells.

**MATERIALS AND METHODS**

Reagents and kits

All primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The BCA protein quantification kit was purchased from Pierce Company (Prod # 23227; Pierce Biotechnology, Rockford, IL, USA). SYBR® Premix Ex Taq™ GC (Perfect Real Time) was purchased from Takara (Code No. RR071A, Dalian, China). Curcumin was purchased from Calbiochem (Catalog # 239802; Lu Jolla, CA, USA), dissolved in DMSO, and stored at −20 °C.

Cell culture

The two prostate cancer cell lines, DU145 and PC3 cells, were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in complete medium (RPMI-1640 medium supplemented with 10% FBS, streptomycin 100 mg/mL, and penicillin 100 U/mL) at 37 °C in a 5% CO₂ humidified incubator.

MTT assay

Cells were seeded with 100 μl of complete medium in 96-well plates (5 × 10³ cells per well). Different doses of curcumin (0, 2, 5, 10, 25, and 50 μM) were added to each well for the indicated durations. MTT reagent (5 mg/mL) was added to each well 48 h after curcumin treatment and incubated for 4 h at 37 °C. Formazan crystals were solubilized by the addition of 100 μL of DMSO. The optical density at 570 nm was measured, and cell viability was determined using 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. Cells were treated with curcumin for the indicated durations, and wound closure was photographed using an inverted microscope equipped with a digital camera. The extent of wound healing 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 the cell proliferation inhibitor mitomycin-C (10 μg/mL). This assay was performed in triplicate and repeated at least three times.

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 Na3VO4) containing a protease inhibitor cocktail. Cell lysates were centrifuged at 13 800 g for 30 min to collect supernatants. Protein concentration was determined using the BCA kit. Total proteins were separated using SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% nonfat milk and incubated overnight at 4 °C with primary antibodies. Blots were washed in PBST (PBS-Tween-20) and incubated for 1 h with horse-radish peroxidase (HRP)-conjugated secondary antibody. Blots were washed in PBST and visualized using enhanced chemiluminescence (Prod # 32106; Pierce Biotechnology, Rockford, IL, USA) followed by exposure to a Fujifilm LAS3000 Imager (Fujifilm Co., Tokyo, Japan). Band densities on the blots were normalized to the relevant β-actin band density using Quantity One software (Bio-Rad Laboratories, Hercules CA, USA).

ChIP assay

Cells were treated with curcumin for the indicated times and fixed with 1% formaldehyde. Nuclei were isolated.
Cells were sonicated, and soluble chromatin DNA was precleared using a protein A bead slurry and salmon sperm DNA. Precleared chromatin was immunoprecipitated overnight with 2 μg of a Notch-1 antibody and normal rabbit IgG as a negative control. Chromatin was extensively washed, de-crosslinked, and purified for routine PCR to detect the target Hes-1 promoter using the following primer pair: forward, 5'-CTGAAAAGTACTGTOGG-3'; reverse, 5'-TGAGCAAGTGCTAGGGG-3'. β-actin (forward 5'-CTGGGACCTCTGTAACAAATGCA-3'; reverse, 5'-AAGGACCTTTCCTGTAACAAATGCA-3') was used as a loading control. qPCR was also performed for quantitative analyses. The ChIP qPCR signals of curcumin treatment were subtracted by the IgG signals. The IgG-normalized ChIP data are presented as a percentage of control (0 h), which was arbitrarily set as 100%.

Statistical analysis

Results in this study are expressed as the mean ± standard deviation (SD). Student’s t-test was used for statistical analyses using SPSS v16.0 software (SPSS Inc., Chicago, IL, USA). Differences of p < 0.05 were considered statistically significant.

RESULTS

Effects of curcumin on prostate cancer cell viability

Two cell lines of prostate cancer, DU145 and PC3, were chosen for our study because they are widely used in in vitro studies of prostate cancer. We examined the effects of curcumin dose and exposure time on cell viability. Different curcumin concentrations (0, 2, 5, 10, 25, and 50 μM) were added to the medium when the cell density reached approximately 50%. MTT assays were performed 48 h later to examine cell viability. Curcumin significantly reduced cell survival of the two cell lines in a dose-dependent manner. Curcumin (10 μM) reduced cell viability of PC3 cells (p < 0.05). The effect was more pronounced in DU145 cells in which 5 μM of curcumin significantly reduced cell viability (p < 0.05). Survival of both cell lines gradually decreased with increasing curcumin concentrations and reaching approximately 20% at 50 μM (Fig. 1). These results demonstrate that curcumin significantly inhibits the viability of DU145 and PC3 prostate cancer cells.

We investigated the time–effect relationship between curcumin and DU145 and PC3 cell viability. Cells were harvested at different time points of 25 μM of curcumin exposure (0, 12, 24, 36, and 48 h), and MTT was performed to detect cell survival. Curcumin (25 μM) significantly reduced the survival of DU145 and PC3 cells in 24 h (p < 0.05). The viability of both cell lines was reduced to approximately 50% 48 h after curcumin treatment (Fig. 2). These results provide further evidence of the inhibitory effect of curcumin on prostate cancer cell viability.

Effects of curcumin on prostate cancer cell proliferation

We used the BrdU incorporation method to detect DNA synthesis and cell proliferation following curcumin treatment and more directly determine the effects of curcumin on prostate cancer cell proliferation. Cells were processed in the same manner as described above, and the proliferation of both cell lines was also examined using dose and time. Curcumin significantly reduced the proliferation of both cell lines in a dose-dependent manner. Curcumin at 5 μM inhibited cell proliferation (p < 0.05). The proliferation rate of both cell lines gradually slowed with increasing curcumin concentrations and
reached approximately 30% at 50 μM (p < 0.05). This effect was dose-dependent (Fig. 3).

Both cells exhibited remarkably reduced proliferation ability 24 h after treatment with 25 μM of curcumin (p < 0.05) and reached 65% at 48 h (Fig. 4).

Effects of curcumin on prostate cancer cell migration

Malignant tumors frequently metastasize, and then we used a scratch assay to examine the effects of curcumin on prostate cancer cell migration. Mitomycin-C is widely used to investigate cell migration in vitro, which exhibits a significant inhibitory effect on cell proliferation, but does not alter cell movement. Scratches were made artificially with pipette tips when the cell density reached 100%, and 25 μM of Curcumin was added to the medium for 24 h. DMSO was added to another group of cells as a control. Curcumin inhibited scratch healing of DU145 cells (Fig. 5A). We measured the width of the scratch and performed statistical analyses to determine the time required to close the scratch. The scratch closed to 40% in control cells treated with DMSO 24 h after treatment (Fig. 5B). Curcumin treatment significantly decreased the healing speed, which reached only 20%. DMSO treatment in the control group in scratch wound healing of the 24-h rate was 40%. There was a statistically significant difference (p < 0.05) between the two groups of cells, which suggests that curcumin inhibited the migration of DU145 prostate cancer cells.

Effects of curcumin on Notch and NICD expression in prostate cancer cells

Numerous studies demonstrated that the Notch signaling pathway was associated with the proliferation and migration of prostate cancer cells. Therefore, we investigated the expression of Notch and its active form NICD following curcumin treatment. DU145 prostate cancer cells were treated with 10 μM and 50 μM of curcumin for 24 h, and cells were collected for Western blot analyses of total protein. Curcumin treatment produced no marked change in Notch-1 and NICD expression.
and the control gene β-actin, which suggests that curcumin cannot affect Notch-1 and NICD expression (Fig. 6).

**Effects of curcumin on MT1-MMP and MMP2 expression in prostate cancer cells**

The results above revealed that curcumin inhibited the migration of DU145 prostate cancer cells, but it had no effect on Notch-1 and NICD expression. Therefore, we inferred that curcumin regulated other genes related to cell migration. Two principal genes in eukaryotic cells, MT1-MMP and MMP2, are involved in the regulation of cell migration, and the expression levels of these proteins positively correlate with cell migration. Therefore, we examined the effects of curcumin on MT1-MMP and MMP2 expression. Curcumin treatment significantly decreased MT1-MMP and MMP2 expression in DU145 cells. The effect of 50 μM of curcumin was significantly greater than 10 μM, which suggests a dose-dependent effect (Fig. 7).

**Effects of curcumin on DNA-binding ability of NICD in prostate cancer cells**

The lack of change in Notch-1 and NICD expression in DU145 prostate cancer cells suggested a role for curcumin in the regulation of the Notch signaling pathway qualitatively, but not quantitatively. Curcumin may regulate the function of NICD. The primary function of NICD is transcription regulation. Therefore, we speculated that curcumin regulates the DNA-binding ability of NICD. We used a chromosome immunoprecipitation assay to examine the DNA-binding ability of NICD in DU145 cells after curcumin treatment.

The DNA-binding ability of NICD was significantly altered in DU145 cells treated with 10 μM of curcumin (Fig. 8). The content of DNA in the promoter region of HES-1 that was co-precipitated with NICD was remarkably reduced. The DNA content in the promoter region of HES-1 was barely detectable following treatment with 50 μM of curcumin, which further reduced the DNA-binding ability of NICD.

We used real-time quantitative PCR to detect the abundance of HES-1 promoter DNA and more accurately determine the inhibitory effects of curcumin on the DNA-binding ability of NICD. As shown in Fig. 9, after treatment, only 53.8% of DNA was detected after treatment with 10 μM of curcumin compared with the control group, and only 14.5% of DNA was detected following 50 μM of curcumin, which is consistent with the PCR results.

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![Fig. 6](image6.png)  
**Fig. 6.** Effects of curcumin on Notch and NICD expressions in DU145 cells using Western blot analyses.

![Fig. 7](image7.png)  
**Fig. 7.** Effects of curcumin on MT1-MMP and MMP2 expressions in prostate cancer cells using Western blot analyses.

![Fig. 8](image8.png)  
**Fig. 8.** The DNA level of HES-1 co-precipitated with NICD in DU145 cells after curcumin treatment using PCR analysis.

![Fig. 9](image9.png)  
**Fig. 9.** The DNA level of HES-1 co-precipitated with NICD in DU145 cells after curcumin treatment by qPCR analysis (six wells/group). “*” represents “p < 0.05”.

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DISCUSSION

Piccolella et al. (10) investigated the effects of curcumin on prostate cancer cells using angiogenesis and cell invasion. ERK1/2 phosphorylation is closely related to VEGF production, and their group investigated the phosphorylation status of ERK1/2 and VEGF expression level in PC3 cells after curcumin treatment. Curcumin significantly inhibited ERK1/2 phosphorylation in PC3 cells, which was accompanied by decreased VEGF expression. These changes partially contributed to the enhanced cell migration ability. Recent studies demonstrated that VEGF and the Notch signaling pathway were closely related (11). Increased expression of the Notch ligand DLL4 significantly suppressed the VEGF-induced proliferation of vascular endothelial cells and inhibited VEGFR expression (12). Specific Notch inhibitors reversed these changes (13). Notch also inhibited the differentiation of endothelial progenitor cells to suppress neovascularization (14). The regulation of angiogenesis by the Notch signaling pathway is not known, but Lan Cao proposed that the Notch signaling pathway played a negative role in the regulation of the functions of VEGF, as reported by a previous study (15).

This study found that curcumin did not directly affect the cleavage of Notch protein, namely NICD production. The Notch signaling pathway functions at two different layers. Cleavage of Notch in the cell membrane is an important prerequisite for activation of the Notch signaling pathway. The intracellular fragment of Notch, NICD, only functions after Notch cleavage. NICD must be transferred to the nucleus to regulate the transcription of target genes that assist with many transcription factors, which is the second layer of regulation in the nucleus. Our data revealed that curcumin affected the Notch signaling pathway primarily in the nucleus. We hypothesize that another mechanism exists in the cytoplasm, in which curcumin regulates the Notch signaling pathway. Activated NICD must pass through the cytoplasm and nuclear membrane to enter the nucleus, and the stability of NICD and its associated chaperones may also be involved in Notch signaling pathway regulation.

Another intriguing finding of this study was that curcumin inhibited the expression of MT1-MMP. MT1-MMP, which is also known as MMP1 (matrix metalloproteinase-14), is a membrane protein in the matrix metalloproteinase family (16). This type of protease dissolves the extracellular matrix and degrades the extracellular matrix and basement membrane, which are important mediators for the invasion and metastasis of malignant tumors (17). One group reported that MT1-MMP was closely associated with the Notch signaling pathway (18). These authors examined the effects of MT deletion in BMSCs (bone marrow stromal cells) on the differentiation of hematopoietic progenitor cells. Their data demonstrated that the functional loss of MT1-MMP led to enhanced Notch signaling pathway activity in the co-cultured hematopoietic progenitor cells and decreased their ability to differentiate into B cells. The Notch-specific inhibitor DAPT inhibited this phenotype. Biochemical studies further demonstrated that MT1-MMP interacted with and cut the Notch ligand DLL1, which is essential for activation of the Notch signaling pathway. Therefore, MT1-MMP may negatively regulate the Notch signaling pathway by cutting DLL1. This study was the first report of MT1-MMP regulation of the Notch signaling pathway via a Notch ligand. The latest study in 2014 demonstrated that MT1-MMP directly cut Notch to regulate Notch ligand DLL1. This study found that MT1-MMP expression correlated positively with the active form of Notch, NICD, in 70% of clinical samples with malignant melanoma. A similar phenotype was also observed in 80% of malignant melanoma cell lines. Further in vitro experiments demonstrated that changes in MT1-MMP expression levels altered Notch signaling pathway activity. They also provided evidence that MT1-MMP colocalized with Notch on the cell membrane, which indirectly suggests that MT1-MMP regulates Notch cleavage. Whether MT1-MMP directly interacts with Notch is not known. Therefore, we cannot exclude the involvement of other molecules, such as the Notch ligand DLL1, in this process. This study found that curcumin did not affect Notch cleavage, but it inhibited MT1-MMP expression. The cells in our study were cultured individually but not co-cultured. Therefore, MT1-MMP could not affect the activity of Notch without the Notch
ligand DLL1, which explains the lack of change in Notch cleavage when MT1-MMP expression was altered.

Notably, our study suggests that MMP2 may be another target for curcumin. Many studies demonstrated that MMP2 was closely related to the development and metastasis of prostate cancer. For example, Chen et al. (20) reported that miR-130b controls prostate cancer cell metastasis via regulation of MMP2 expression. The nuclear receptor TR4 also promotes prostate cancer metastasis via the positive regulation of MMP2 expression (21). Lacorte et al. (22) also found that calcium balance in the extracellular matrix was associated with MMP2 expression in prostate cells. Fibronectin regulates the survival of prostate cancer cells via MMP2. Finasteride is a clinical drug for the treatment of prostate cancer, and this drug remarkably suppresses the survival of prostate cancer cells accompanied by a downregulation of MMP2, which suggests that finasteride inhibits prostate cancer via the regulation of MMP2 activity (23).

REFERENCES