

Curcumin Alters the Migratory Phenotype of Nasopharyngeal Carcinoma Cells through Up-regulation of E-Cadherin

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Abstract. Background: Curcumin is a natural polyphenol. It is a potent suppressor of nuclear factor kappa B (NF- κ B). High NF- κ B levels have suppressive effect on E-cadherin (molecule related to cell–cell adhesion) in nasopharyngeal carcinoma (NPC) cells. We hypothesized that suppressing NF- κ B by curcumin could up-regulate E-cadherin expression in NPC cells. Materials and Methods: NPC cell lines HK1 and HONE1 were used. Real-time quantitative PCR and Western blotting were used to examine the expression changes of NF- κ B and E-cadherin. A mouse xenograft model was used to validate the results. Results: With curcumin treatment, NF- κ B was down-regulated and E-cadherin was up-regulated in NPC cells. The negative correlation of NF- κ B and E-cadherin was confirmed in the mouse xenograft model. Conclusion: Our results suggest that curcumin could be used in preventing NPC migration by suppressing NF- κ B and activating E-cadherin expression.

Nasopharyngeal carcinoma (NPC) is an epithelial cancer arising from the epithelial surface covering the nasopharynx. In our locality, undifferentiated NPC is the major histological type (1). One of the unique characteristics of undifferentiated NPC is its association with Epstein–Barr virus (2). NPC is a highly invasive cancer with high metastatic potential (3). The expression of viral protein encoded by Epstein–Barr virus further increase the invasiveness of NPC (4).

Curcumin (diferuloylmethane) is a polyphenol isolated from the rhizome of *Curcuma longa*, a common dietary spice

called turmeric (5). Purified curcumin is a yellowish powder and is widely used as herbal medicine in China and India. Emerging evidence suggests that curcumin is a potent therapeutic agent for cancer. Curcumin has been shown to have anti-inflammatory, antimetastatic, antioxidative, and antiproliferative effects on cancer cells (6).

The diverse function of curcumin is associated with its potent inhibitory effect on nuclear-factor kappaB (NF- κ B). NF- κ B was shown to promote cancer development (7) and metastasis (8). Simiantonaki *et al.* frequently detected nuclear NF- κ B localization in cells dissociated from the surrounding cohesive cancer tissues. They suggested that nuclear localization of NF- κ B is associated with loss of adhesion of cancer cells (9). In NPC, overexpression of NF- κ B is frequently observed (10). However, the link between NF- κ B and NPC migration is not clear.

E-Cadherin, or type 1 cadherin, is a calcium-dependent cell–cell adhesion glycoprotein. E-Cadherin is a major player in cell–cell adhesion of epithelial cells and acts as metastatic suppressor in epithelial carcinomas (11). Loss of E-cadherin is common in NPC and is significantly associated with advanced diseases (12). A recent study revealed that E-cadherin suppression is associated with NF- κ B up-regulation in epithelial cancer (13). The up-regulation of NF- κ B induced expression of transcription inhibitors and prohibited E-cadherin transcription (14).

Since curcumin is a potent inhibitory agent of NF- κ B, we believed that curcumin treatment could up-regulate E-cadherin expression in NPC. We first tested the effects on NPC cell lines HONE1 and HK1 and validated the results in a xenograft model.

Materials and Methods

Cell cultures and reagents. NPC cell lines HONE1 and HK1 were used. All the NPC cells were provided by Professor SW Tsao (Department of Anatomy, The University of Hong Kong). All cell lines were maintained in RPMI-1640 medium (Gibco, NY, USA)

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supplemented with 10% fetal bovine serum (Gibco), 200 Unit/ml penicillin G sodium (Gibco), 200 µg/ml streptomycin sulfate (Gibco), and 0.5 µg/ml amphotericin B (Gibco). Curcumin (Sigma-Aldrich, St. Louis, MO, USA) was first dissolved in absolute ethanol to a final concentration of 40 mM and was stored at -20°C before use. The dissolved curcumin was diluted to working concentrations with RPMI-1640.

Cytotoxicity test. The effective range of curcumin was determined by *in vitro* Toxicology Assay Kit Sulforhodamine B (SRB) assay (Sigma-Aldrich) following the suggested protocol of the manufacturer.

RNA extraction and real-time polymerase chain reaction (RT-PCR). Total RNA was extracted by Trizol (Invitrogen, USA). First-strand cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The E-cadherin (*CDH1*) and *NF-κB* levels were evaluated by real time RT-PCR using an ABI 7900TH Fast Real-time PCR system (Applied Biosystems, CA, USA). Expression levels were evaluated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$).

Immunohistochemical staining of cultured cells. Cells were grown on coverslips and were treated with an appropriate concentration of curcumin for 48 hours. After treatment, cells were fixed with 2% paraformaldehyde/PBS (pH 7.2) (International Laboratory, USA). Non-specific proteins were blocked with 2% bovine serum albumin (Sigma-Aldrich). The slides were incubated with anti-E-cadherin mouse monoclonal antibody (Zymed Laboratories, CA, USA) or anti-NF-κB p65/Rel A mouse monoclonal antibody (Upstate Biotechnology, NY, USA) at 1:100 dilution. Visible color signal was developed using Dako EnVision+ System-HRP (DAB) (Dakocytomation, CA, USA) according to the manufacturer's suggested protocol.

Western blotting. Cells were lysed with cell lysis buffer containing 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.01% PMSF and 0.02% protease inhibitor (Roche protease inhibitor cocktail) for 30 minutes on ice. Protein concentration was determined by BCA Protein Assay Kit (Pierce Biotechnology, IL, USA). SDS-PAGE (10% for NF-κB and 6% for E-cadherin) was performed using Mini-protein III system (Bio-Rad, Hercules, CA, USA). Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corporation, Massachusetts, NE, USA) by semi-dry transfer cell (Bio-Rad). Membranes were incubated with anti-E-cadherin monoclonal antibody (Zymed Laboratories) at 1:4,000 dilution or anti-NF-κB p65/Rel A monoclonal antibody (Upstate Biotechnology) at 1:5,000, dilution or beta-actin specific antibody (Sigma-Aldrich) at 1:20,000 dilution at 4°C overnight. Signals were developed using chemiluminescence (ECL) Plus Western Blotting Detection System (Amersham, UK) according to the instructions of the supplier.

Cell migration assay. To determine the migration ability, a scratch wound assay was performed. The culture surface was coated with fibronectin (16 µg/ml). Cells were seeded to form a nearly confluent cell monolayer on the culture surface. Wounds were then made by using sterile pipette tips and the cell debris was removed by washing with PBS several times. The wounded cell monolayer was then incubated with cell culture medium containing an appropriate concentration of curcumin.

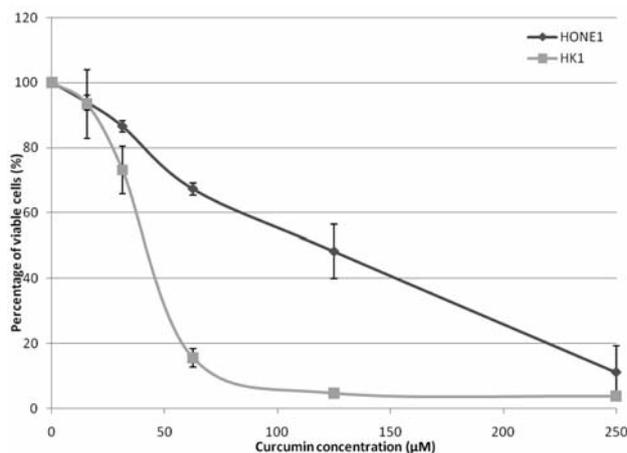


Figure 1. Dose-dependent cytotoxic effects of curcumin on NPC cells. NPC cells were treated with curcumin (0-250 µM) for 48 hours. The experiment was performed in triplicate. Data are expressed as means±SD.

Animal models. Female athymic *nu/nu* mice (5 weeks old, weight range: 18-22 g) were used. Mice were maintained under pathogen-free conditions under controlled temperature and humidity. All animal experiments were performed at the Animal Laboratory in Department of Surgery of the University of Hong Kong. The protocol for animal study was approved by Institutional Committee on the Use of Live Animals in Teaching and Research (Protocol number 1388-06). A total of 2×10^6 HONE1 cells (viability 95-97%) in 100 µl RPMI-1640 medium were injected subcutaneously into the right flank of mice. Tumor growth was measured daily with calipers in two dimensions. Tumor volume was calculated using the formula: Volume (in mm³)=(L × W²)/2, where L is the length in millimeters and W is the width in millimeters. When the tumor volume reached 150 mm³, the mice were randomly assigned into two groups. In group 1 (n=4), 25 µl of curcumin in absolute ethanol (150 mg/kg) were injected into the tumor center three times per week. The treatment lasted for 1 month. In group 2, 25 µl of absolute ethanol alone was injected into the tumor center at the same frequency for 1 month. Tumor volume was measured every day with calipers. After 30 days of treatment, mice were sacrificed with an excessive dosage of pentobarbital. The subcutaneous tumors were removed, fixed and subjected to immunohistochemical staining.

Hematoxylin and eosin staining. The tumor sections were dewaxed with xylene and rehydrated through a descending series of ethanol. The sections were then stained with hematoxylin for 5 minutes and washed with distilled water. With incubation in Scott's tap water for a few seconds, the sections were washed in distilled water again. Sections were then stained with eosin for 20 seconds and washed in distilled water. Finally, sections were rehydrated through an ascending series of ethanol, cleared in xylene for 5 minutes and mounted.

Immunohistochemical staining of xenograft. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded section (4 µm). The sections were first dewaxed with xylene. Antigen retrieval was achieved by microwaving in 10 mM sodium citrate buffer (pH 6.0) for 15 minutes. To block the endogenous peroxidase activity, the sections were incubated in 3% hydrogen peroxide for 10

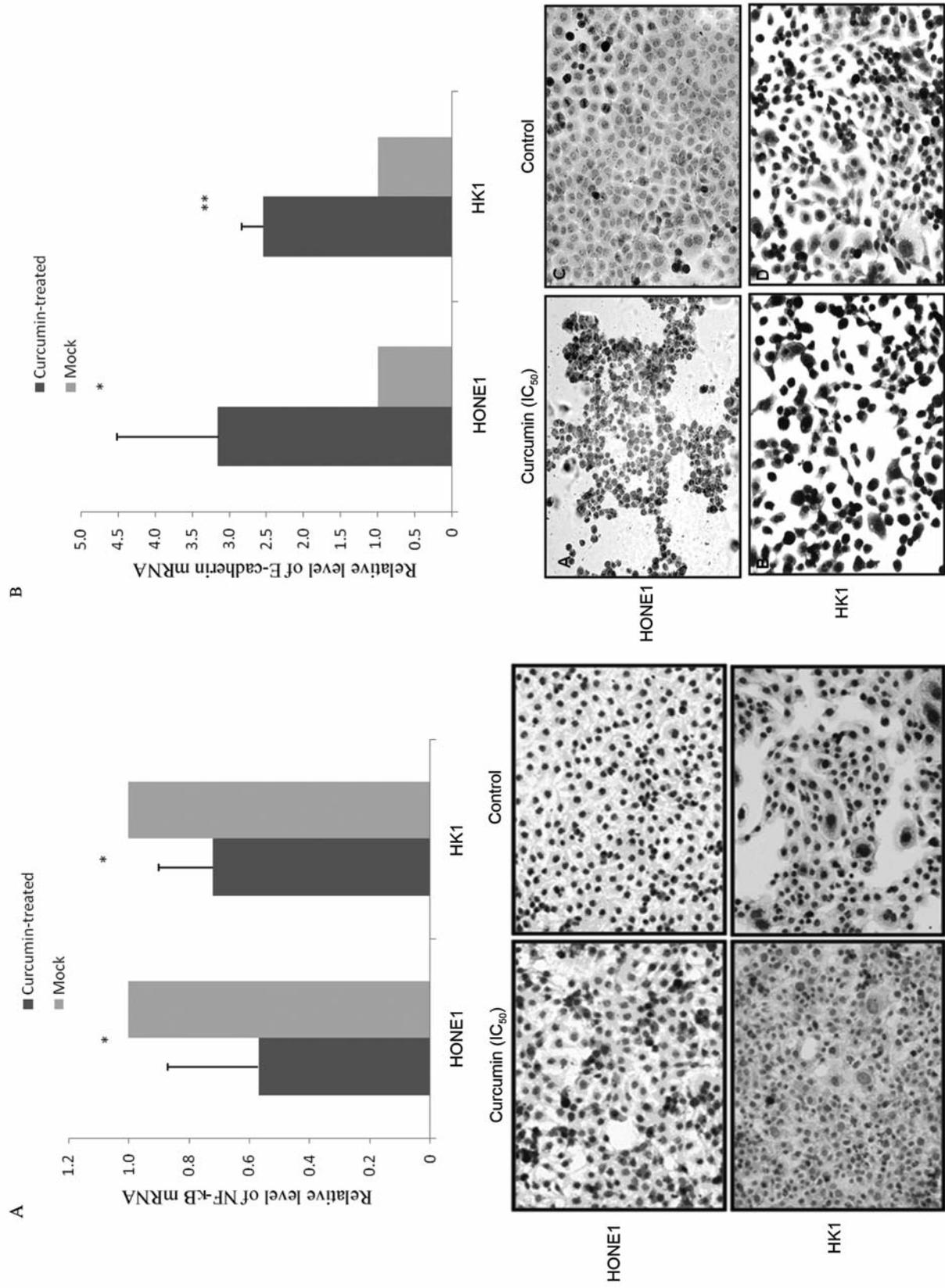


Figure 2. Expression changes of NF-κB (A) and E-cadherin (B) in NPC cells in response to curcumin treatment. NPC cells were treated with curcumin at IC₅₀ for 48 hours. Expression of genes is given as the expression level relative to the house-keeping gene 18S. The experiment was performed in triplicate. *p-Value <0.05; ***p-value <0.01.

minutes. The slides were stained with E-cadherin-specific antibody (Zymed Laboratories) or NF- κ B p65/Rel A antibody (Upstate Biotechnology) at 1:100 dilution. Signals were developed with Dako EnVision+ System- HRP (DAB) (Dakocytomation).

Statistical analysis. Data analysis was performed using SPSS® for Windows version 14.0 (SPSS, Inc., Chicago, IL, USA). All data were two-sided. *P*-values <0.05 were considered as statistically significant.

Results

Effective dose of curcumin on NPC cells. We first determined the effective dose of curcumin on HONE1 and HK1 cells by sulforhodamine B assay (Figure 1). *In vitro* cytotoxic effects were observed when the cells were treated with curcumin at a concentration ranging from 15-250 μ M. The IC₅₀ (defined as the drug concentration which caused 50% reduction of cell viability) of HONE and HK1 cells was 125 μ M and 47 μ M respectively.

NF- κ B expression in NPC cells. NF- κ B mRNA and protein were detected in HONE1 and HK1 cells (Figure 2A). At IC₅₀, reduction of NF- κ B mRNA was observed in both HONE1 and HK1 cells (HONE1: 1.78-fold reduction, *p*=0.03; HK1: 1.42-fold reduction, *p*=0.02). NF- κ B protein was localized in the nucleus of HONE1 and HK1 cells. Cytoplasmic NF- κ B protein was not detected in either of the two cell lines. The quantity of nuclei-located NF- κ B was reduced with curcumin treatment. The degree of reduction was revealed by Western blotting analysis (Figure 3). The suppressing effect induced by curcumin was more prominent in HK1 cells in comparison with HONE1 cells.

E-Cadherin expression in NPC cells. E-Cadherin mRNA and protein was detectable in HONE1 and HK1 cells (Figure 2B). In response to curcumin, E-cadherin mRNA and protein were up-regulated. At IC₅₀, an increase in E-cadherin mRNA levels was observed in both HONE1 (3-fold increase, *p*=0.05) and in HK1 (2.5-fold increase, *p*=0.008) cells. In Western blotting analysis, E-cadherin-inducing effects were observed in both HONE1 and HK1 (Figure 3).

Effects of curcumin on NPC cell migration. Scratch wound healing assay was performed to assess the effects of curcumin on NPC cell migration. At 24 h, a significant proportion of HONE1 and HK1 cells had migrated to the wound created. Conversely, cell migration was not observed in NPC cells treated with curcumin (Figure 4).

Curcumin suppressed NF- κ B and elevated E-cadherin levels in mouse xenograft. Having established that curcumin suppressed NF- κ B and up-regulated E-cadherin expression in NPC cell lines, we further validated the effects in a mouse model. We used HONE1 to generate the xenograft model. We

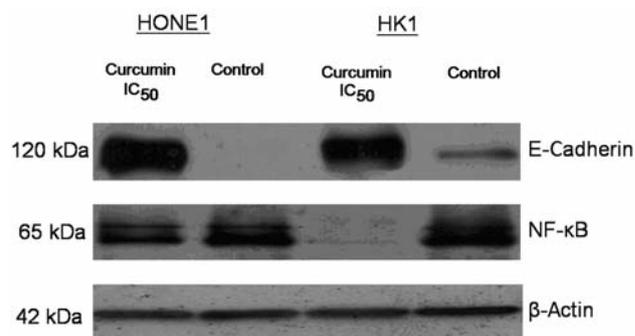


Figure 3. Western blot analysis showing changes in expression level of NF- κ B and E-cadherin after curcumin treatment. With curcumin treatment, the NF- κ B (65 kDa) level decreased and E-cadherin (120 kDa) level increased in both lines. Beta-actin (42 kDa) was used as an internal control to standardize the protein loading.

treated the xenograft with a low-dose of curcumin to prevent tissue necrosis induced by a high-dose of curcumin. As shown in the H&E staining (Figure 5 upper panel), the injected curcumin did not induce necrosis in the xenograft. In comparison with the control, curcumin-treated xenograft had an obviously higher E-cadherin level. Additionally, NF- κ B protein levels were significantly reduced in the xenograft.

Discussion

Curcumin is a potent inhibitor of NF- κ B in head and neck cancer (15). However, its exact mechanisms of action are not clear. Curcumin was shown to prevent activation of NF- κ B induced by TNF α , phorbol esters and hydrogen peroxide through suppressing the I κ B kinase activity. The inactivation prevents the entry of NF- κ B into the nucleus and thus reduces the expression of cell cycle regulatory proteins (16, 17). Curcumin can down-regulate NF- κ B in numerous types of cancer including breast, colorectal, leukemia, ovarian, and pancreatic (18-22). However, until the present study, the effects of NF- κ B had not yet been evaluated in NPC.

In the present study, we first demonstrated that curcumin suppressed transcriptional expression of NF- κ B in the NPC cells. Two epithelial NPC cell lines were included in this study, HONE1 and HK1. HONE1 cell line was derived from poorly differentiated NPC (23) and HK1 was derived from well-differentiated NPC (24). Inhibitory effects of curcumin were observed in both cell lines. Both immunohistochemical staining and Western blot analysis indicated that expression of NF- κ B is suppressed by curcumin treatment.

E-Cadherin reduces cell-cell adhesion and increase the motility of tumor cells (25). E-Cadherin is frequently down-regulated or lost in most NPC. Loss of E-cadherin expression is associated with invasion and metastasis of NPC (12). Simiantonaki *et al.* showed that loss of E-cadherin was associated with up-regulation of NF- κ B expression (13). We

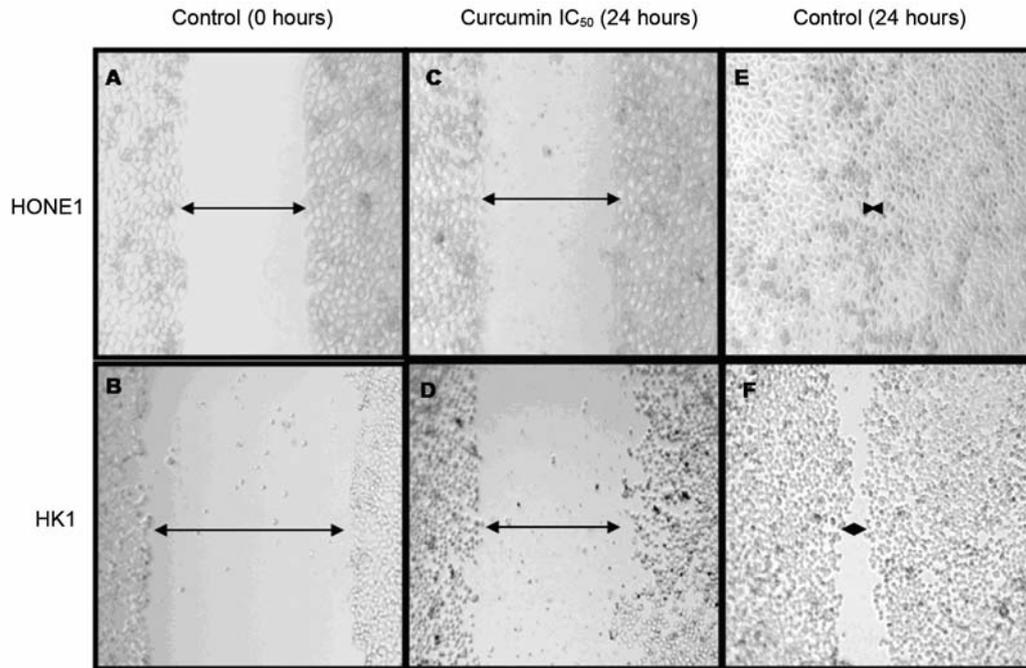


Figure 4. Scratch wound assay on NPC cells. The migration ability of NPC cell lines was assessed during the assay. A and B: The construction of a wound after near confluence of HONE1 and HK1 cells, respectively. With 24 hours of incubation, C and D: curcumin-treated groups showed inhibition of migration of both cell lines. With the same incubation time, E and F: control groups showed a faster migration rate, achieving a complete or near closure of the wounds of HONE1 and HK1 cells, respectively.

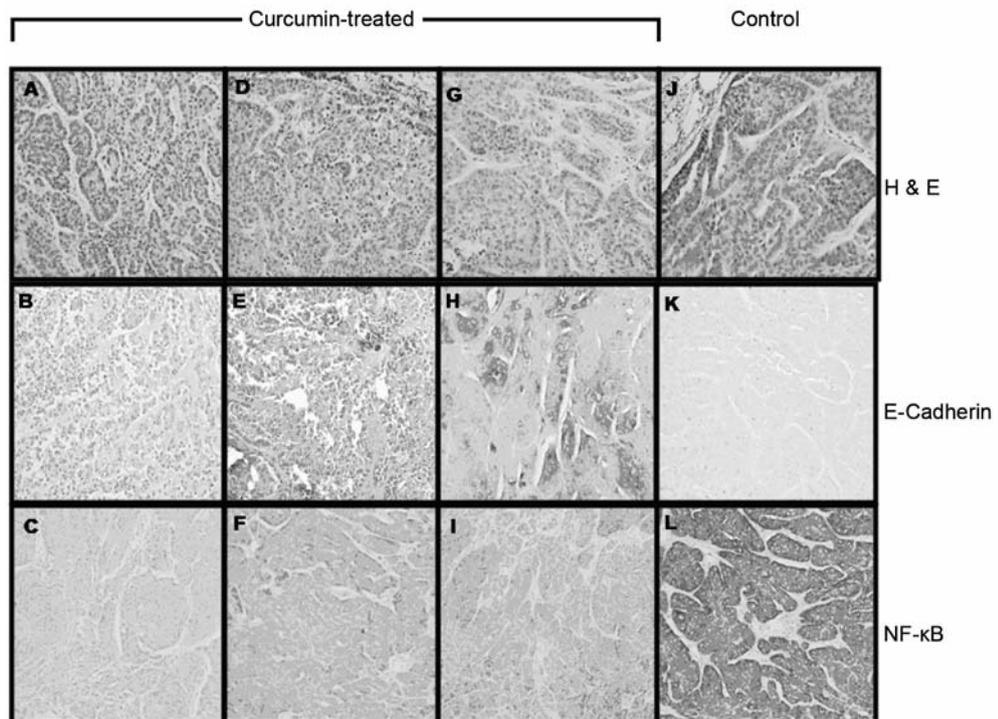


Figure 5. Immunohistochemical staining of NF- κ B and E-cadherin on mouse xenograft. Immunohistochemical analysis of xenograft. B, E, H and K: Sections were stained with antibodies specific for E-cadherin; C, F, I and L: Sections were stained with antibodies for NF- κ B. Magnification: $\times 200$.

therefore hypothesized that E-cadherin levels should increase if NF- κ B could be suppressed effectively. Our results demonstrate the negative correlation between NF- κ B and E-cadherin expression in NPC and their expression was effectively reversed by curcumin treatment.

In summary, with curcumin treatment, a decrease of NF- κ B expression and increase of E-cadherin was observed in NPC cells. Moreover, curcumin was able to reduce NPC cell migration, presumably through E-cadherin reactivation. Since the use of curcumin is generally safe, the potential use of curcumin as a chemopreventive or chemotherapeutic agent warrants further investigation.

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