Research Article

Cancer Prevention Research

Curcumin Inhibits Carcinogen and Nicotine-Induced Mammalian Target of Rapamycin Pathway Activation in Head and Neck Squamous Cell Carcinoma

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Abstract

Curcumin appears to be a safe, bioactive food compound that is a potential chemopreventive for patients at a high risk for head and neck squamous cell carcinoma (HNSCC). Identification and validation of intermediate endpoints is an important step in evaluating chemopreventive agents. AKT/MTOR pathway biomarkers are intrinsic to the carcinogenic process as well as the mechanism of intervention with curcumin. Antiproliferative effects of curcumin were assayed in 9 HNSCC and a keratinocyte cell line. Nicotine, a genotoxic alkaloid involved in tobacco addiction, forms DNA adducts and has been implicated in upper aerodigestive tract cancer promotion. The antiproliferative effects of curcumin were associated with inhibition of the AKT/MTOR pathway in presence and absence of nicotine, which also induced this pathway. Curcumin was highly effective at suppressing growth of SCC40 xenografts and its activity is associated with modulation of MTOR's downstream target pS6. Curcumin at 15 mg significantly increased survival (286 ± 37 vs. 350 days) in the 4NQO carcinogenic model survival study. A major cause of lethal progression of HNSCC is local regional migration and invasion of malignant cells, and curcumin significantly inhibited cancer cell migration and invasion *in vitro* and *in vivo* where downregulation of pS6 was associated with a significant decrease in MMP-9. This is the first study to demonstrate that curcumin inhibits the adverse effects of nicotine by blocking nicotine-induced activation of the AKT/MTOR pathway in HNSCC, which retards cell migration. These studies indicate that inhibiting the AKT/MTOR pathway with curcumin may be useful as an oral chemopreventive agent. *Cancer Prev Res; 3(12); 1586–95. ©2010 AACR.*

Introduction

Annually, there are ~40,000 new head and neck squamous cell carcinoma (HNSCC) with 13,000 U.S. deaths and 500,000 new cases worldwide (1). Cigarette smoking alone still accounts for 30% of all U.S. cancer deaths, in spite of smoking prevalence reductions (2). Despite treatment advances, overall survival rates (~45%) have not improved significantly over the last 3 decades (3). Ninety percent to 95% of diagnosed head and neck malignancies are tobacco-related (4, 5). Tobacco use at HNSCC diagnosis is a recognized risk factor for second primary tumors (SPTs), and current smokers are 3 times more likely than never-smokers to develop smoking-related SPT (6), indicating a need for chemopreventive agents among tobacco users. One promising agent is curcumin.

Intermediate endpoints are necessary to more rapidly assess interventions for primary cancer prevention and address the feasibility posed by large patient numbers, length of study, and cost when cancer occurrence or recurrence is an endpoint (7). Signaling pathways upstream and downstream of MTOR appear dysregulated in greater than 90% of HNSCC (8) and we have shown overexpression of EIF4E in tumor-free surgical margins is an independent predictor of recurrence (9) and is activated by the AKT/MTOR pathway (10). In earlier studies, as the degree of dysplasia increased, there was an increase in EIF4E overexpression in surgical margins of HNSCC patients (11). Hence, AKT/MTOR pathway activation in premalignant oral cavity lesions could provide an important intermediate endpoint in chemopreventive agent trials. Previous curcumin clinical trials for other organ sites have tested NF-κB and its downstream effects as curcumin biomarkers (12).

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Nicotine, one of the active tobacco product components, activates the AKT/MTOR pathway contributing to carcinogenesis by inducing cell survival, proliferation, and angiogenesis (13, 14). Because curcumin is believed to inactivate MTOR (15) possibly through downregulation of AKT signaling (16), we want to determine if curcumin modulates the AKT/MTOR pathway, a leading molecular target for cancer and correlate AKT/MTOR modulation with its biological consequence.

A major cause of lethal HNSCC progression is local regional migration and invasion of malignant cells. The MTOR protein complex integrates a variety of intracellular signaling pathways often dysregulated in cancer (8) making MTOR inhibition an attractive antitumor target. MTOR is activated by PI3K-AKT signaling, and in a negative feedback loop, links MTOR inhibition to activation of the PI3K-AKT pathway (17) through MTOR-p70S6K–mediated inhibition of IRS1. MTOR activity regulates the 2 best studied targets S6K1 that phosphorylates the 40S ribosomal protein S6, and phosphorylates 4EBP1 through EIF4E (18).

The EIF4E translation initiation factor regulates transcription of weak mRNAs with highly structured 5′ untranslated regions (UTR) including growth-promoting proteins directly related to tumorigenesis and is activated through the AKT/MTOR pathway. AKT/MTOR pathway inhibition inhibits 2 important downstream targets p70S6 kinase (S6K) and the 4E-binding protein (4EBP1; ref. 19). When 4EBP1 is inactivated, unphosphorylated 4EBP1 binds to EIF4E. The decrease in free EIF4E levels leads to a decrease in cap-dependent translation of specific mRNAs, such as cyclin D1, c-myc, b-FGF, VEGF, and MMP-9 (matrix metalloproteinases) thus leading to inhibition of cell proliferation, angiogenesis, and invasion (9, 20). MMPs are a family of zinc-dependent endopeptidases involved in extracellular matrix degradation and play a significant role in extracellular matrix invasion (21), and increased MMP-9 expression is linked to EIF4E overexpression, resulting in the invasive phenotype (9).

Curcumin's molecular mechanism of action and primary cellular targets and corresponding biological significance are not clearly defined. A natural product isolated from turmeric, curcumin has been implicated as a powerful therapeutic in a variety of human cancers (22) and is currently undergoing clinical trials for colon, skin, pancreatic, and hematologic cancers. On the basis of curcumin's ability to block tumor initiation and progression in other cancers, we examined its effects on HNSCC growth in a variety of HNSCC cell lines, xenograft model, and in a carcinogen-induced survival study. We wanted to determine whether curcumin has growth inhibitory effects in HNSCC and not in normal keratinocytes, and whether curcumin's growth inhibitory effects are a result of AKT/MTOR pathway inhibition. We then determined whether curcumin inhibited nicotine-induced AKT/MTOR pathway activation and migration to mimic the clinical situation where up to 50% of head and neck cancer patients continue to use tobacco after treatment of their primary malignancy (5). These studies indicate a mechanism by which curcumin can suppress invasion and metastasis and may represent a target for HNSCC chemoprevention and treatment.

Materials and Methods

Curcumin

Curcumin C3 Complex (>98% pure) was obtained from Sabinsa Corp. *In vivo* studies were conducted with curcumin suspended in saline paste for oral application, or in corn oil for oral gavage feeding. Doses ranged from 5 to 15 mg/day.

Cell lines

Cell lines obtained from ATCC: FaDu and SCC25, and OKF6 (oral keratinocytes) were provided by Anil Rustgi (University of Pennsylvania Medical Center). SCC40, SCC066, SCC114, SCC116 were obtained from Susanne M. Gollin (University of Pittsburgh), and PCI13, PCI15a, and PCI30 were provided by Teresa Whiteside (University of Pittsburgh). SCC40, SCC066, SCC116, and FaDu are hyperdiploid for PTEN (23, 24). Cancer cell lines were maintained as previously described (23). OKF6 cells were cultured in media containing human keratinocyte growth supplement (Invitrogen). All chemicals were obtained from Sigma-Aldrich.

Cell proliferation

Around 2,000 cells per well were seeded in triplicate onto 96-well plates in complete media at 37°C with 5% CO_2. After adherence, cells were treated with curcumin (0–40 μmol/L), nicotine (0.001–10 mmol/L), or both for 0 to 72 hours. Three HNSCC cell lines were also cultured in oral keratinocyte media containing human keratinocyte growth factors to confirm effects of curcumin on the HNSCC cell lines irrespective of growth media. Cell viability was measured using MTS (Promega).

Flow cytometry

Cells were grown to ~80% confluency in the absence or presence of 10 to 20 μmol/L curcumin for 0 to 48 hours, as significant growth inhibition was noted at this dose. DNA was stained with propidium iodide after RNAse A treatment to remove RNA and analyzed by FACS Caliber (Becton Dickson).

Subcutaneous HNSCC xenograft model

Studies were conducted in compliance with Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee guidelines. Animals housed in a barrier facility were maintained on a normal diet *ad lib*. In the xenograft model, 4-to-6-week-old Balb/c nu/nu mice (Harlan) were injected subcutaneously with 1×10^6 (in 100 μL saline) SCC40 cells in each flank. To determine whether curcumin inhibits tumor formation after engraftment of cells, treatment was initiated when tumors reached 40 mm^3 (defined as day 0), and treated with curcumin by daily oral gavage (0, 5, 10, or 15 mg of curcumin in 100 μL of corn oil). The SCC40 tongue cell line was chosen as this

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location represents 20% to 40% of human oral cancers. Tumor volume [(length × width^2)/2] was determined by digital caliper and tumors were harvested at necropsy. Mice were monitored daily for adverse effects and as a surrogate marker of toxicity, body weight was measured daily without any differences observed between treated and control animals.

To determine the chemopreventive effects of curcumin prior to grafting, ten 4-to-6-week-old Balb/c nu/nu mice per group were pretreated for 4 days with curcumin by daily oral gavage (0, 10, or 15 mg of curcumin in 100 μL of corn oil) and injected subcutaneously with 1×10^6 (in 100 μL saline) SCC40 cells in each flank. Mice continued to receive curcumin for the remainder of the study and the control group received vehicle only. Tumor volumes were compared on day 24, the last day when all control mice were still alive.

Oral carcinogen-induced model

Twenty-four 4-to-6-week-old CBA/CaJ mice received a low-dose (5 mg/mL) tobacco-derivative 4-nitroquinoline 1-oxide (4NQO) painted in their oral cavity (100 μL) 3 times per week for 20 weeks that mimics tobacco-induced carcinogenesis in humans (25), and followed up to 50 weeks. During the same period of carcinogen exposure, mice were also painted 5 times per week with 0, 5, 10, or 15 mg curcumin (in 100 μL of corn oil) to determine if curcumin prevents carcinogenesis. Mice were monitored for adverse effects and body weight was measured daily without any differences observed between treated and control animals. All mice were sacrificed upon oral tumor formation, where tumor-free survival was determined by the number of weeks surviving before oral tumor formation.

Transwell migration assay

Cell motility was determined by transwell migration. HNSCC cells (1×10^5) were seeded in upper wells of precoated (collagen 3 mg/mL) transwell membranes (8-μm pore size) in serum-free media containing curcumin (0–20 μmol/L). The same media as the upper wells was placed in the lower wells with addition of the chemoattractant lysophosphatidic acid (100 nmol/L) or nicotine (0.01 or 0.1 mmol/L), or nicotine (0.1 mmol/L) with curcumin (0–20 μmol/L) and incubated for ∼18 hours. Cells on the upper well and membranes coated with collagen were swabbed to mechanically remove nonmigrating cells, and cells attached to the bottom of the membranes were fixed with methanol, and stained with crystal violet. Six fields per concentration were counted for cells attached to the lower surface of the filter under a light microscope. Data represent mean ± SEM pooled from at least 3 independent experiments. The number of cells in different media, representing the migration level with differing curcumin concentrations was compared.

Nicotine-induced activation of HNSCC cells

HNSCC cell lines SCC40, PCI15a, FaDu, and SCC066 were serum-starved overnight and pretreated with 20 μmol/L of curcumin (2 hours) and/or 100 μmol/L of nicotine for the final 30 minutes and analyzed by western blot analysis in triplicate.

Western blot analysis of treated cells and tumors

Soluble proteins extracted from cell lines and tumor cells from the *in vivo* experiments in the established tumor model were analyzed by Western blot (10). The following antibodies were used: rabbit polycolonal primary antibodies from Cell Signaling: 4E binding protein 1 (4EBP1; 1:500), phospho-4EBP1 (Thr37/46; p-4EBP1; 1:250), S6 ribosomal protein (1:100), phospho-S6 ribosomal protein (Ser235/236; 1:100), AKT (1:250), phospho-AKT (Ser473; 1:250), caspase-3 (1:1,000), MMP-9 (1:500 dilution), and actin (1:3,500). Mouse monoclonal cyclin D1 (1:100) and rabbit polyclonal FGF-2 (147; b-FGF; 1:100) were obtained from Santa Cruz Biotechnology.

Immunohistochemical assessment of oral tongue and tumors

Tongues harvested at necropsy, embedded in paraffin and hematoxylin and eosin (H&E) stained, were verified by our study pathologist for degree of dysplasia. Xenograft tumors were also stained with CD31 (Santa Cruz Biotechnology) as described (26), and for AKT/MTOR pathway inhibition with pS6 (1:100) obtained from Cell Signaling. Slides were read and scored by the study pathologist (FA) blinded to the study. For each antibody, no staining was scored as [−], weak or focal staining as [+], intermediate staining as [++], and strong staining as [+++]. Scoring was further simplified by considering only ++ and +++ as positive staining whereas negative and focal staining as negative. Digital images were acquired to obtain the average pS6 immunoreactivity of 25 cells per field from 3 separate fields for each tumor ($n = 5$ mice per group). To examine curcumin's effects on angiogenesis, the number of blood vessels was determined (27).

Statistics applied for the analysis

Proliferating cell percentage was compared using 1-way analysis of variance (ANOVA). Tukey's multiple comparison test to determine the differences among individual treatment groups. ANOVA or Kruskal–Wallis was used to determine significant differences. A Tukey's multiple comparison as a post-hoc test was performed to evaluate differences among treatment groups. For the tumor-free survival study, a Kaplan–Meier survival curve with log-rank test was used to compare all groups.

Results

HNSCC cell lines are more sensitive to curcumin when compared with normal cell lines

To determine whether HNSCC cell lines are sensitive to curcumin, a cell proliferation assay was performed on 9 HNSCC cell lines and a keratinocyte cell line. Analysis was significant [$F(3,44) = 123.29$, $P < 0.001$], and HNSCC cells were more sensitive to curcumin with significant inhibition

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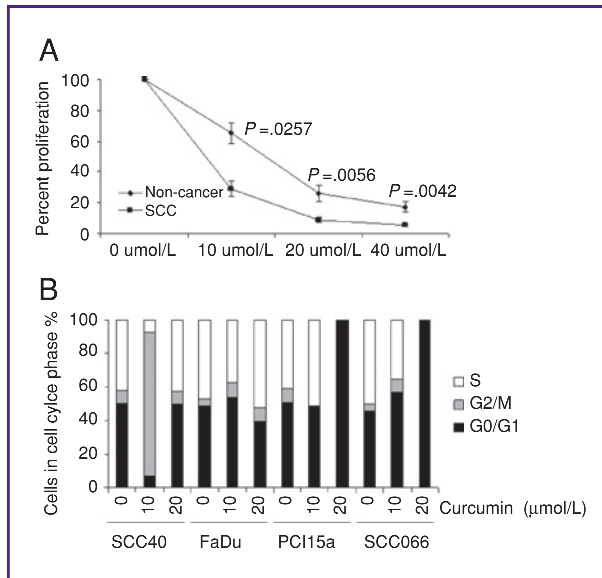

Fig. 1. Curcumin inhibits HNSCC cell proliferation. A, cell viability. After adherence, cells were treated with curcumin for 48 to 72 hours. Average percent proliferation of noncancer cell line (keratinocyte) and cancer (SCC) cell lines ± SEM. B, fluorescence activated sorting (FACS) analysis of HNSCC cells treated with 0, 10, or 20 μmol/L of curcumin for 24 to 48 hours. Note the shift from G1 phase to G2 phase for SCC40 at 10 μmol/L, and the G1 shift in PCI15a and SCC066 at 20 μmol/L.

at 10 μmol/L of curcumin compared with oral keratinocytes (OKF6; Fig. 1A and Supplementary Fig. 1A) after 72 hours curcumin treatment. Because normal cell lines grow in different media, concurrent studies for curcumin's effects on HNSCC cells grown in oral keratinocyte media containing human keratinocyte growth factors were conducted in parallel with MEM media. No difference was obtained with PCI15a (Supplementary Fig. 1B) or SCC40 (Supplementary Fig. 1C) cell lines when cultured in the oral keratinocyte media compared with MEM media.

The decreased cell growth induced by curcumin could be attributable to increased apoptosis or decreased cell-cycle progression. Therefore, we studied the effects of curcumin on the cell cycle. The percentage of apoptotic cells in the sub-G1 peak did not increase or convincingly demonstrate a cell-cycle arrest with curcumin treatment in most of the cell lines tested at 10 μmol/L, with the exception of the significant ($P < 0.001$) G2/M shift in SCC40 (Fig. 1B). However, at 20 μmol/L, 2 cell lines (PCI15a and SCC066) demonstrated a G1 cell-cycle arrest. These results indicate curcumin inhibited cell proliferation but did not induce apoptosis in HNSCC cell lines studied under these conditions. Because curcumin has been shown to induce a G2/M cell-cycle arrest in colon cancer (28), we focused on the SCC40 tongue cell line for our *in vivo* studies as this location represents 20% to 40% of human oral cancers.

Curcumin inhibits growth and delays carcinogenesis in mice

Curcumin appears to slow growth compared with control in SCC40 xenograft tumors after tumor cells had a

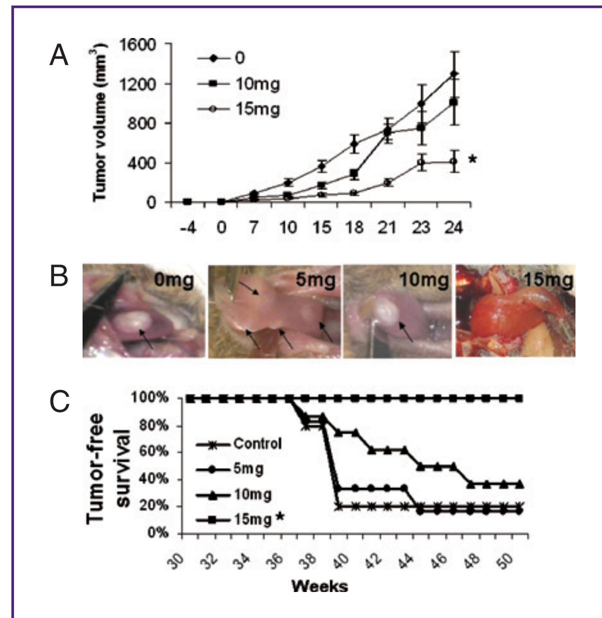

Fig. 2. *In vivo* activity of curcumin. A, mice were pretreated with the indicated dose of curcumin for 4 days prior to injection with 1 × 106 SCC40 tumor cells in each flank (day 0) and continued receiving daily curcumin treatment (5 mice per group, mean tumor volume ± SEM, *, $P = 0.007$ vs. control group). B, locally delivered curcumin prevents oral tumor formation in carcinogenesis-induced model. Representative oral tumors in 4NQO-treated mice. Arrows demonstrate lesions on all aspects of the tongue surface, except in the 15 mg group where no tumors were noted. C, tumor-free survival curves for control and curcumin-treated groups; *, $P < 0.001$ versus control group.

chance to engraft (Supplementary Fig. 2A). Using the Wilcoxon rank-sum test, there was a significant difference in average tumor volumes between control and treatment groups at 5 mg of curcumin for only the early time points (days 0–16, $P = 0.02$ for 5-mg dose) when tumors were of smaller volumes. When repeated at a higher dose, there was a significant difference in tumor volume between control and 15 mg of curcumin again at early time points, day 5 ($P = 0.01$) and day 8 ($P = 0.02$), but not on day 12 ($P = 0.7$) or day 14 ($P = 0.8$; Supplementary Fig. 2B). No significant difference was noted at 10 mg curcumin.

The difference of tumor growth and proliferation between curcumin and control groups in the established tumor model led us to explore the role of curcumin in preventing tumor formation of the same HNSCC cell line. Curcumin pretreatment was highly effective at suppressing growth of SCC40 xenograft tumors as evidenced by the difference in tumor volumes between control (vehicle-treated) and the 15 mg treated group of mice [$F(4,7) = 34.92$, $P = 0.007$]. No difference was noted at 10 mg of curcumin (Fig. 2A). No toxicity was observed with total body weights remaining stable throughout the experiment.

Curcumin inhibited oral tumor growth compared with control in the carcinogen-induced model. No visible tumors formed in the highest curcumin dose (15 mg) group. Mice in the lower dose groups (0, 5, and 10 mg)

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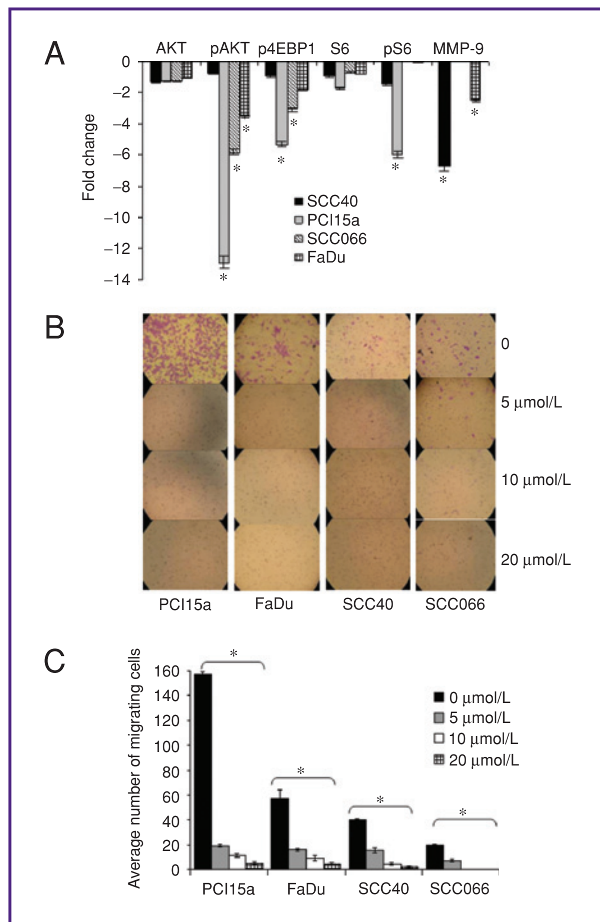

Fig. 3. Curcumin inhibits AKT/MTOR pathway and migration and invasion potential. A, whole cell lysates from serum-starved (18 hours) HNSCC cells were treated with vehicle or 20 µmol/L of curcumin for 24 hours and subjected to Western blot analysis with the indicated antibodies. Optical density values were calculated and compared after normalizing to actin. Mean ± SD fold-expression level change of 3 separate experiments with or without curcumin, *, $P < 0.01$ versus control group. B, representative bright-field images of cells treated with vehicle or curcumin at the indicated concentration were captured at 200× magnification from 3 to 5 independent experiments. C, quantification of HNSCC cell migration mean, ± SEM. * $P < 0.001$ versus control group.

developed at least 1 large tumor, and 1 mouse in the 5 mg curcumin treatment group developed multiple small tumors (Fig. 2B). Mice were sacrificed upon tumor formation or 50 weeks if tumors did not develop. All mice treated with the highest dose (15 mg) of curcumin survived (the entire 50 weeks, $P < 0.001$) without visible tumors compared with mice in the control and lower curcumin doses (286 ± 37 days for control, 289 ± 34 days for 5 mg, and 313 ± 37 days for 10 mg) indicating curcumin delays carcinogenic transformation (Fig. 2C).

Curcumin inhibits gene products modulated by the MTOR pathway *in vitro*

We used 4 HNSCC cell lines to investigate curcumin's effects on the AKT/MTOR pathway *in vitro* with 20 µmol/L of curcumin for 24 hours as inhibition of proliferation or migration was noted at this dose and time point (Fig. 3A). Total AKT levels were unaffected ($P = 0.6$) whereas pAKT was inhibited ($P = 0.003$). Total S6 levels were unaffected ($P = 0.8$) whereas pS6 was inhibited only in PCI15a ($P < 0.05$). However, in FaDu cells, pS6 was inhibited with prolonged treatment at 48 and 72 hours ($P < 0.01$).

Because curcumin inhibited the AKT/MTOR pathway, we wanted to determine curcumin's effects on the migration potential of HNSCC through weak mRNAs translationally regulated by EIF4E that is MMP-9. There was an average 4.6-fold decrease in MMP-9 expression with curcumin in 2 cell lines that expressed MMP-9 (Fig. 3A). Compared with vehicle, curcumin significantly inhibited migration of PCI15a, SCC40, FaDu, and SCC066 (Figs. 3B and C). A significant difference was found among the 4 curcumin concentrations for all 4 cell lines ($P < 0.001$). Similarly, the number of cells in control media was significantly higher than the number of cells in curcumin media ($P < 0.001$), and the number of cells in all 3 curcumin-treated groups was not significantly different ($P > 0.05$) in all 4 cell lines.

Curcumin blocks effects of MTOR induction by nicotine

Activated AKT initiates a cascade of signaling events involved in oncogenic transformation. PCI15a and SCC40 were treated with nicotine in the absence or presence of curcumin, and effects on the AKT/MTOR pathway were assayed (Fig. 4). Curcumin alone did not inhibit expression of total AKT ($P = 0.6$) or significantly inhibit AKT phosphorylation at 20 µmol/L ($P = 0.1$) when exposed to curcumin for only 2 hours in SCC40 cells (Fig. 4A compare lanes 1 and 2 "no curcumin" and lanes 3 and 4 "with curcumin"), and PCI15a cells (compare lanes 1 and 2 "no curcumin" and lanes 3 and 4 "with curcumin"). MTOR is activated by a variety of signaling pathways and curcumin's effects on MTOR's downstream effectors were more pronounced than on pAKT when exposed to 20 µmol/L for only 2 hours (Fig. 4B). Rapid curcumin-dependent inhibition of S6 phosphorylation and 4EBP1 phosphorylation (lanes 1 and 2 "no curcumin" and lanes 3 and 4 "with curcumin") was noted. A more pronounced effect on the MTOR pathway was observed in PCI15a compared with SCC40 cells where nicotine activated downstream effectors of the MTOR pathway as demonstrated by nicotine-induced expression of pS6 and p4EBP1 in PCI15a but not pS6 in SCC40 (compare lanes 1 and 2 "no nicotine" and lanes 5 and 6 "with nicotine"). More importantly, curcumin inhibited MTOR pathway activation irrespective of nicotine in SCC40 and PCI15a (compare lanes 3 and 4 "with curcumin" and lanes 7 and 8 "with nicotine and curcumin").

The AKT/MTOR pathway integrates a variety of intracellular signaling pathways involved in cell-cycle progression, proliferation, invasion, and angiogenesis through expression of the weak mRNAs' cyclin D1, MMP-9, and b-FGF. Curcumin decreased cyclin D1 ($P < 0.01$) and b-FGF ($P = 0.001$) expression (Fig. 4A) in SCC40 cells and cyclin D1

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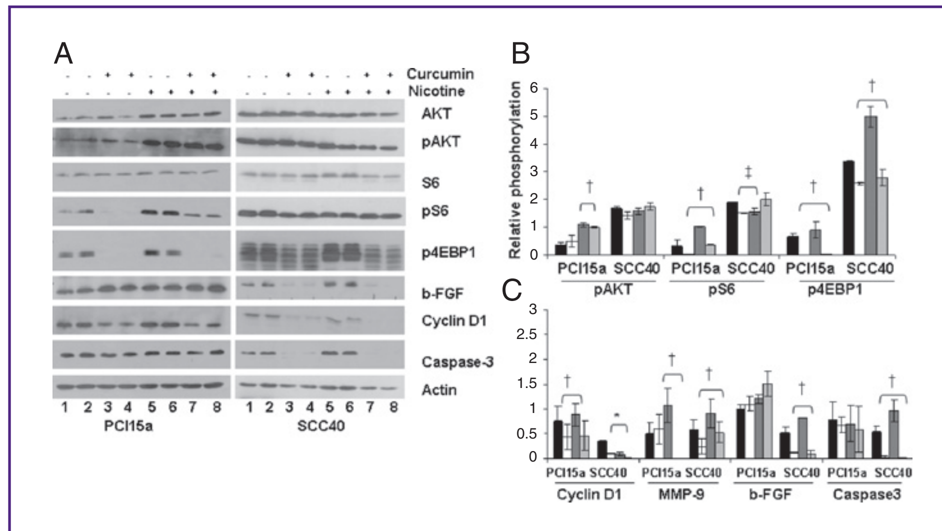

Fig. 4. Curcumin suppresses nicotine-induced MTOR pathway activation. A and B, serum-starved (18 hours) PCI15a and SCC40 cells were pretreated with 20 μmol/L of curcumin (lanes 3, 4, 7, and 8) or vehicle (lanes 1, 2, 5, and 6) for 2 hours. For the final 30 minutes, 100 nmol/L of nicotine (lanes 5–8) or vehicle (lanes 1–4) was added. Whole-cell lysates were subjected to Western blot analysis with the indicated antibody. B, quantification of relative expression levels of the indicated markers with nicotine and curcumin. Whole-cell lysates were subjected to Western blot analysis with the indicated antibody and quantified. Control, black columns; curcumin, white columns; nicotine, dark gray columns; curcumin and nicotine, light gray columns; mean expression level of 4 separate experiments, ± SEM (‡, $P < 0.05$, †, $P < 0.01$ and *, $P < 0.001$ compared with control).

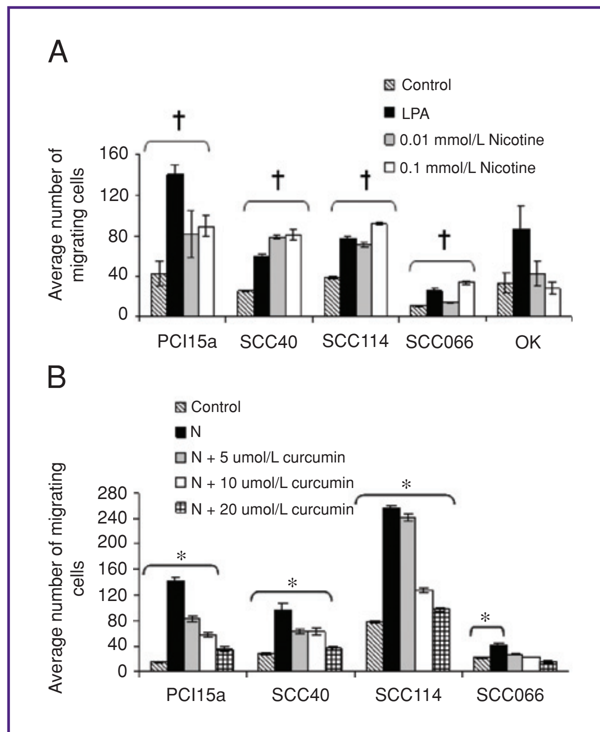

Fig. 5. Curcumin suppresses nicotine-induced invasion and migration A, HNSCC (PCI15a, SCC40, SCC114 and SCC066) and normal oral keratinocyte (OK) cells were treated with vehicle, lysophosphatidic acid (LPA) as a positive control, or nicotine at the indicated concentration (†, $P < 0.01$ compared with control). B, HNSCC cells were treated with vehicle, nicotine (N, 0.1 mmol/L), and curcumin at the indicated concentration (*, $P < 0.001$ compared with control), mean ± SEM.

($P = 0.01$) in PCI15a (compare lanes 1 and 2 "no curcumin" and lanes 3 and 4 "with curcumin"). MMP-9 was not initially inhibited by curcumin alone in the highly invasive PCI15a cell line, although curcumin completely abrogated MMP-9 in nicotine-activated PCI15a and significantly inhibited MMP-9 in SCC40 (Fig. 4C). The short duration and curcumin concentration used was not sufficient to induce apoptosis in PCI15a as demonstrated by the lack of caspase-3 cleavage, but did induce cleavage in SCC40, suggesting curcumin's effects on invasion and angiogenesis are its main targets.

Because curcumin inhibited MMP-9 in nicotine-activated cells, we explored curcumin's effects on the nicotine-induced migration potential of HNSCC. Compared with vehicle, nicotine significantly activated migration of HNSCC cell lines ($P < 0.01$) but not normal oral keratinocytes (Fig. 5A), and curcumin inhibited the nicotine-induced migration of HNSCC ($P < 0.001$, Fig. 5B). Cancer cell migration permits dissemination from the primary tumor, and sensitivity of HNSCC cells to retardation of cell migration by curcumin in the absence or presence of nicotine further highlights its chemopreventive properties.

Curcumin inhibits gene products modulated by the MTOR pathway *in vivo*

We next evaluated curcumin's effects on the MTOR pathway in xenograft tumors. Curcumin treatment inhibited phosphorylation of S6 ribosomal protein at Ser235/236, downstream of MTOR, compared with control treatment in xenograft tumors (percentage of pS6 positive cells, 95% CI: 14.4–17.4 with curcumin and 65.4–83.9 without curcumin, $P < 0.001$; Fig. 6A). Curcumin reduced MMP-9

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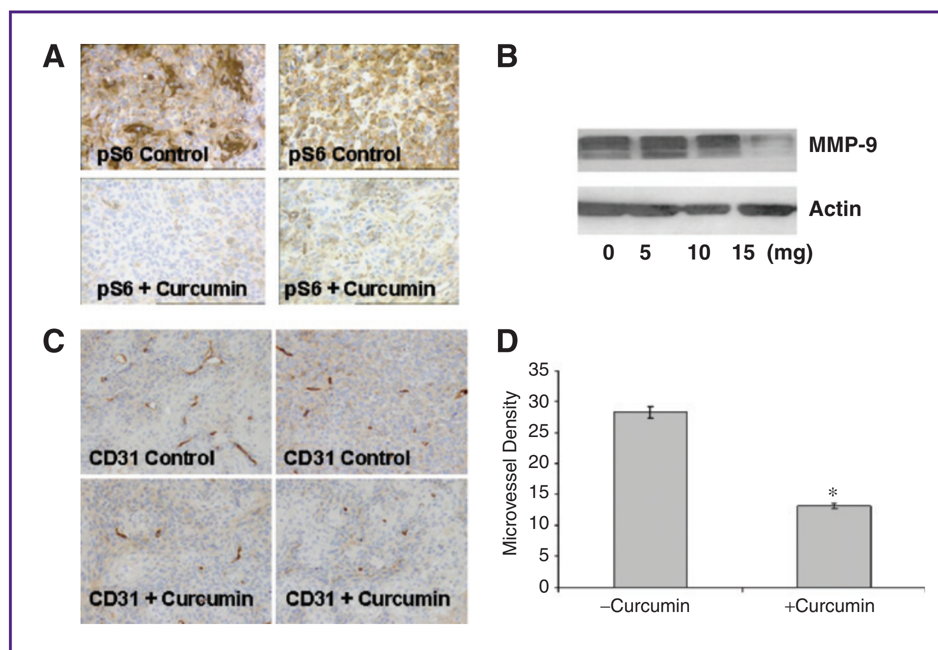

Fig. 6. Curcumin inhibits gene products modulated by the MTOR pathway *in vivo*. A, representative intense reddish-brown staining of pS6 expression in xenograft tumors from mice ($n = 5$ mice per group) treated with control (-Curcumin) and faint brown cytoplasmic staining from mice treated with 15 mg curcumin shown at 200× magnification. B, representative Western blot analysis of MMP-9 expression in xenograft tumors from mice treated with the indicated curcumin dose. C and D, for vessel density quantification, tumor sections from control and 15 mg curcumin treatment group were stained with anti-CD31 antibody. The intense reddish-brown stained CD31+ microvessels are shown at 200× magnification. D, microvessel density (MVD) from control and 15 mg curcumin treatment group were counted at ×400 magnification, mean ± SEM *, $P < 0.001$ versus control group.

expression in a dose-dependent manner (Fig. 6B). Inhibition of b-FGF in the *in vitro* experiment noted in the SCC40 cell line led us to investigate curcumin's effects on angiogenesis through CD31. We noted a significant decrease in microvessel density (Figs. 6C and D, 95% CI: 15.5–13.6 with curcumin and 26.0–30.4 without curcumin, $P < 0.001$).

Discussion

Up to 50% of patients with advanced stage disease develop a recurrence of HNSCC. Therefore, preventing any step of neoplastic transformation is a promising strategy. Patients that continue smoking after successful HNSCC treatment carry a 3-fold increased risk of SPT whereas smoking cessation is associated with a 50% risk-reduction of developing oral cancer within 3 to 5 years (6). In a placebo-controlled randomized chemoprevention trial of 13-*cis*-retinoic acid (6), 70% of observed SPTs were smoking-related lesions. Only 16% of current smokers successfully stopped smoking over the 10-year follow-up period, suggesting patients who continue to smoke after successful HSNCC treatment carry a higher risk of developing tobacco-induced SPTs. This is the first study to demonstrate curcumin inhibits the adverse effects of nicotine by blocking nicotine-induced activation of the MTOR pathway that blocked cell motility.

Previous HNSCC xenograft studies delivered curcumin via topical paste, intratumoral injection or intravenous liposomes (29, 30). We chose to mimic a clinical trial using a locally delivered microgranular curcumin that is currently undergoing clinical trials including one at our institution for HNSCC patients (NCT01160302). A previous study demonstrated curcumin inhibited carcinogen bioactivation in oral SCC (31). For this study, we investigated the effects of nicotine in the presence of curcumin, as nicotine replacement therapy has become prevalent in tobacco cessation, and potential head and neck cancer chemopreventive agents should be evaluated in the presence of nicotine.

Our *in vitro* data consistently demonstrated sensitivity of SCC cells to curcumin with significant inhibition of cell proliferation as low as 10 μmol/L of curcumin compared with oral keratinocytes. This suggests the possibility of a favorable therapeutic window *in vivo*. It is striking that a variety of cell lines display a wide range of sensitivity to MTOR inhibitors, including rapamycin (sirolimus) and RAD001 (everolimus; 32, 33), whereas a variety of cell lines are consistently sensitive to curcumin within a narrow range at physiologically relevant concentrations as noted in our study and others (17).

The above effects led us to explore the role curcumin plays in the cell cycle. The mammalian cell cycle is tightly regulated and divided into the DNA synthetic (S phase) and mitotic (M phase) phases that are immediately preceded by gap phases (G1 and G2). Aberrant regulation can either block cell division or induce hyperproliferation (cancer). Deregulation of cyclin D1, a rate-limiting factor

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in G1 progression, occurs frequently in cancer through its overexpression. Curcumin induces a cell-cycle arrest ranging from G2/M in colon cancer to G0/G1, depending on cell type (34). It would appear that unlike other cancer cell lines, the G2/M arrest observed in SCC40 at the lower curcumin concentration is not a generalized mechanism in HNSCC. The lack of a sub-G1 cell population suggests curcumin does not induce apoptosis at concentrations that inhibit proliferation. Unlike chemotherapeutic agents given at toxic doses to induce apoptosis of cancer cells, successful chemopreventive agents are nontoxic to cells; yet often possess anti-inflammatory and antioxidant properties.

Pharmacokinetic studies indicate low curcumin bioavailability following oral administration. A common approach to improve bioavailability has been large daily dosing of curcumin up to 8 to 12 grams that demonstrated no dose-limiting toxicity (35). Limited data has been published on the *in vivo* effects of curcumin given in a controlled oral dose. One study demonstrated 4 mg of daily curcumin alone was sufficient to suppress AKT phosphorylation but not sufficient to suppress breast cancer xenograft proliferation (36), whereas 10 mg daily curcumin by oral gavage inhibited NF-κB activation and suppressed ovarian xenograft proliferation (37). This highlights the need to carefully select an effective *in vivo* curcumin dose. Curcumin appears to work more effectively in the pretreatment model whereas in the established tumor model, its growth inhibitory effects were noted only at earlier time points, which may suggest curcumin inhibits the grafting of tumor cells but once tumors are established, curcumin has minimal effects and probably higher doses may be required to overcome bioavailability. However, as noted in the true chemoprevention experiment with 4NQO, curcumin significantly improved tumor-free survival, providing further evidence that curcumin inhibits earlier stages of tumor development. We show curcumin delays carcinogenesis at the highest dose tested in mice indicating curcumin's potential role as a chemopreventive agent or as adjuvant therapy to prevent recurrence. The significant difference in the 15 mg curcumin treatment group compared with control demonstrates curcumin has growth inhibitory effects and prevents tumor formation in an oral carcinogen-induced model. The dose required for bioactive food compounds is an important question and a higher dose of curcumin was required to prevent tumor formation.

Cancer cell migration is a highly regulated process that permits dissemination from the primary tumor. The invasive phenotype in malignant cells can be inhibited by decreasing MMPs; ref. 38) and MMP-9's aberrant expression in HNSCC has been linked to enhanced tumor invasion or metastasis (9, 39). Curcumin decreased migration and invasion of endothelial cells, non–small cell lung cancer, colorectal cancer, hepatocellular carcinoma, and migration of vascular smooth muscle cells is inhibited with the same concentrations of curcumin by blocking MMP-9 (40). In our study, concentrations that inhibited cell proliferation were sufficient to inhibit migration of HNSCC cell lines. MMP's are critical for invasion of basement membranes; therefore, sensitivity of HNSCC cells to retardation of cell migration by curcumin further highlights its chemopreventive properties. SPT and recurrences are often a result of conversion of premalignant to malignant lesions. This process of dysplasia to carcinoma *in situ* to invasion requires invasion of the basement membrane and angiogenesis. Blood vessels supply nutrients for tumor growth, whereas MMP's are critical for invasion of basement membranes, both important in the carcinogenic process. Dose-dependent inhibition of MMP-9 as noted in our *in vivo* study and decreased blood vessel formation highlights curcumin's usefulness as a chemopreventive agent.

No reliable biomarker exists for clinical application of head and neck cancer. Therefore, we wanted to establish biomarkers for future chemoprevention trials with curcumin. A previous clinical trial demonstrated the AKT/MTOR pathway can be modulated with pathway-specific inhibitors (41), and we show inhibition of cell proliferation by curcumin is accompanied by inhibition of this pathway. The fact that curcumin modulates this pathway is exciting for future clinical trials in prevention. We showed in this study a significant inhibition of AKT and MTOR's downstream targets pS6 and p4EBP1 at low concentrations (Figs. 3 and 4). Curcumin blocks tumor initiation and progression by interfering with multiple pathways. As curcumin acts on so many different targets in different cell types, it is important to determine if curcumin directly affects a few major targets that indirectly impacts downstream factors or if curcumin acts on a multiplicity of downstream targets that might explain different effects in different cell types. Curcumin downregulates the well-established marker NF-κB in a HNSCC cell line (42), and we show the more sensitive markers for head and neck tumors that is AKT/MTOR effectors were modulated with curcumin. This possibly suggests that NF-κB and molecular targets of the AKT/MTOR pathway are useful intermediate endpoints for future chemoprevention trials with the safe, well-tolerated bioactive food compound curcumin.

To correlate the biological effects of curcumin's suppression of gene products regulated by the MTOR pathway, we evaluated MMP-9 and b-FGF at a dose that inhibited migration and proliferation. Both gene products were important for conversion of premalignant to a malignant phenotype and demonstrate that curcumin suppresses the invasive and possible angiogenic potential through MTOR pathway inhibition in HNSCC. Head and neck cancer is characterized by persistent activation of the AKT/MTOR pathway, and we show here that curcumin inhibits nicotine-induced activation of the MTOR pathway and its downstream targets, which inhibited HNSCC proliferation and migration.

Many cancer-promoting events activate the MTOR pathway, leading to increased angiogenesis, migration, and invasion. We hypothesize the anticarcinogenic effects of curcumin are a result of MTOR pathway inhibition and targets of this pathway may be intermediate endpoints in

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future curcumin studies for patients with precancerous lesions of the upper aerodigestive tract (UADT). A large international study by the NIDCR using tissue microarrays in HNSCC noted that unlike EGFR and p53, pAKT and pS6 were activated in greater than 90% of tumors (43). Future studies will establish whether NF-κB and MTOR are sensitive biomarkers of curcumin chemoprevention for recurrence or second primaries in patients with HNSCC prior to embarking on chemoprevention trials in patients with precancerous lesions of the UADT. This is the first study to demonstrate curcumin inhibits nicotine's adverse effects in HNSCC and future studies will establish the mechanism of curcumin's protective effects in nicotine-induced activation of the MTOR pathway *in vivo*. A recent study confirmed curcumin's antimotility activity in an oral SCC cell line (44); however, there is no available information to address curcumin's effects on migration and invasion of HNSCC in the presence of nicotine. Because curcumin is generally well tolerated, does not have significant toxicities, and can be easily administered orally, administration of curcumin in HNSCC patients holds promise.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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