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In Vitro Growth Suppression of Renal Carcinoma Cells by Curcumin

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Purpose
Malignant clear cell renal carcinoma (ccRCC) is an aggressive tumor highly resistant to chemotherapy and radiation. Current therapeutic approaches to management of ccRCC have not significantly improved patient survival, therefore novel therapies are needed. Activated NFκB and STAT3 expression is associated with ccRCC pathogenesis. The dietary polyphenol curcumin is a well-documented antitumor agent and a known inhibitor of NFκB and STAT3 activation. Given the lack of effective therapies that block ccRCC progression, our objective was to examine whether curcumin could suppress the growth and migration of ccRCC cells, and whether this suppression was mediated via inhibition of NFκB and STAT3 activity.

Methods
Human ccRCC cell lines (769-p, 786-o, Caki-1, ACHN and A-498 cells) were exposed to curcumin to assess the impact of curcumin on ccRCC cell viability. Colony formation assay was used to assess the effect of curcumin on ccRCC cell renewal capability. Effect of curcumin on apoptosis was determined by annexin V binding and mitochondrial membrane depolarization assays. The anti-migratory effect of curcumin on ccRCC cells was assessed using the wound healing assay. Effect of curcumin on NFκB and STAT3 phosphorylation in 769-p cells was determined by western blot analysis.

Results
In ccRCC cells, curcumin decreased cell proliferation and cell viability, abolished clonogenic property, induced apoptosis and blocked cellular migration. The growth suppressive and pro-apoptotic effects of curcumin were accompanied by decreased phosphorylation of NFκB and STAT3.

Conclusions
The ability of curcumin to induce apoptosis and inhibit migration of ccRCC cells justifies additional mechanistic and preclinical studies that examine the effect of curcumin or other NFκB and STAT3 inhibitors as potential suppressors of ccRCC tumorigenesis. (J Patient-Centered Res Rev. 2015;2:156-164.)

Keywords
curcumin, dietary polyphenol, apoptosis, renal carcinoma

The incidence of malignant clear cell renal carcinoma (ccRCC) has risen in the last two decades, particularly in African Americans, Hispanics and women. The standard cytokine-based therapy with interferon-α (IFN-α) or interleukin-2 has not been effective at suppressing ccRCC growth (5-year survival less than 10%), and toxicity is common.¹ Although the use of targeted therapies in the clinical management of ccRCC has provided benefits in terms of prolonging progression-free survival, eventually patients develop resistance and disease recurs.²-⁴ Therefore, more effective and less toxic therapeutic agents are needed.

At the molecular level, disruption of several signaling pathways has been shown to contribute to the development and progression of ccRCC. Mutations in the tumor suppressor von Hippel-Lindau gene (VHL) are common in ccRCC.⁵ ⁶ The protein product of VHL, pVHL, functions to suppress transcriptional activity of hypoxia-inducible factor (HIF)-1α and HIF-2α. HIFs regulate the expression of genes involved in cell survival and angiogenesis (transforming growth
factor α, platelet-derived growth factor and vascular endothelial growth factor). Thus, the consequence of an inactive pVHL is increased growth factor expression and growth factor receptor-mediated signaling. The increase in receptor-mediated signaling leads to the activation of transcription factors, including NFκB and STAT3, which have well-defined roles in promoting tumor growth.

Increased NFκB activity stimulates the expression of gene proteins (bcl-2, MMP-2 and MMP-9) critical to tumor survival and invasion. Indeed, increased NFκB activity has been correlated with ccRCC progression, and upregulation of the prosurvival protein bcl-2 in human ccRCC is a common occurrence. The highly metastatic and invasive nature of ccRCC has been attributed to increased expression of matrix metalloproteinases (e.g. MMP-2 and MMP-9) that promote the disruption of extracellular matrices. Expression of bcl-2, MMP-2 and MMP-9 is regulated by NFκB.

Constitutively activated STAT3 is associated with tumor progression, angiogenesis and development of chemotherapy/radiotherapy resistance. These effects of STAT3 are believed to be mediated through upregulation of cyclins, vascular endothelial growth factor and bcl-2. Increased STAT3 expression has been detected in human ccRCC, and STAT3 was shown to promote interleukin-6–mediated proliferation of ccRCC cells.

NFκB and STAT3 are activated by HIF-induced growth factors and play critical roles in promoting ccRCC tumorigenesis. To the best of our knowledge, successful pharmacological targeting aimed at suppressing HIF-mediated transcription in ccRCC has not been achieved. For this study we investigated whether the dietary polyphenol curcumin, a known suppressor of NFκB and STAT3 transcriptional activity, would be effective at suppressing ccRCC tumor cell growth.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Curcumin was purchased from LTK Laboratories (St. Paul, MN) and Sigma-Aldrich (St. Louis, MO). 786-o, Caki-1, ACHN and A-498 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). 769-p cells used in this study were either provided by Dr. A. Sorokin (Medical College of Wisconsin, Milwaukee, WI) or purchased from ATCC. Phospho-p65 NFκB (S536), total-p65 NFκB, phospho-STAT3 (S727) and total-STAT3 antibodies were purchased from Cell Signaling Technology (Danvers, MA).

**Cell Culture and Assessment of Cell Viability**

769-p, 786-o, Caki-1, ACHN and A-498 cells were maintained in complete growth medium containing 10% fetal bovine serum (FBS). Medium was replaced every 2–3 days and subculturing done as required. To assess cell viability, equal number of cells were seeded in a 96-well plate in complete growth medium. After an overnight incubation at 37°C, cells were exposed to vehicle or curcumin in medium containing 0.5% FBS. In experiments that evaluated the dose-dependency of curcumin on ccRCC cell viability or on DNA synthesis, indicated cell lines were exposed to curcumin (0–200 µM) for 72 hours. In experiments that examined the time-course of curcumin-induced suppression of ccRCC cell viability, 769-p cells were exposed to curcumin (0, 5 or 50 µM) for 1, 2 or 3 days. After treatment, cell viability was quantitated by using either the colorimetric MTT assay or the Cell Titer-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) as previously described. DNA synthesis was measured by determining bromodeoxyuridine (BrdU) incorporation into DNA as previously described.

**Colony Formation Assays**

Colony formation ability of 769-p cells was determined as previously described. To briefly summarize, 769-p cells growing in log phase were seeded at a density of 3,000 cells/well in a 6-well plate in complete growth medium. Cells were allowed to adhere for an overnight period, following which the medium was replaced with complete medium containing curcumin (0, 5, 20, 50, 100 or 200 µM). Cells were cultured for approximately 2 weeks, with a medium change (containing fresh vehicle or curcumin) performed every 4–5 days. Crystal violet was used to stain and visualize the colonies.

**Annexin V Staining Assays**

769-p cells were treated with curcumin (0, 5 or 50 µM) for 24 hours. After treatment, cells were washed with phosphate-buffered saline and stained with annexin V-FITC and propidium iodide. Subsequent flow cytometry was performed as previously described.
Western Blotting
769-p cells were treated with curcumin (0, 5, 20 or 50 µM) for 30 minutes. After treatments, protein content was determined in the cellular lysates and an equal amount of protein subjected to electrophoresis, followed by western blotting with the indicated antibodies as previously described. The pixel intensities of the western blot bands (p-p65 NFκB, t-p65 NFκB, p-STAT3 and t-STAT3) were quantitated using the intensity plot and area calculation functions of the ImageJ program available from the National Institutes of Health (http://rsbweb.nih.gov/ij). After normalization to the band intensities of total proteins (t-p65 NFκB and t-STAT3), p-p65 NFκB and p-STAT3 protein expressions were calculated. The control (vehicle-treated sample) was assigned an arbitrary value of 1, and fold change in the curcumin-treated groups was calculated.

Mitochondrial Membrane Depolarization Assays
769-p cells were treated with curcumin (0, 5 or 50 µM) for 24 hours. Cells were washed, stained with JC-1 dye, and then analyzed by flow cytometry, according to the manufacturer’s protocol (Cell Technology, Fremont, CA) as previously described.

Wound Healing Assay
Wound healing assay for migration was performed as previously described. Briefly, 769-p cells were grown to monolayer confluency in 6-well plates. A sterile 200 pipette tip was used to inflict a scratch wound. Cell debris was removed by washing, and subsequently phase contrast microscopy was used to capture an image of the wound. Impact of 24-hour curcumin treatment (0, 5 or 50 µM) on wound closure in 769-p cells was assessed.

RESULTS
Curcumin Inhibits ccRCC Cell Proliferation and Viability
We initially investigated whether curcumin was effective in suppressing the growth of ccRCC cell lines. To address this, we evaluated the concentration-dependent effect of curcumin on cell viability in a panel of human ccRCC cell lines. 769-p, 786-o, Caki-1, ACHN and A-498 cells were treated with curcumin and cell viability assessed. Our data demonstrated that in all five human ccRCC cell lines, curcumin was able to significantly (P<0.05) suppress cell viability (Figure 1), with maximal (>90%) growth suppression in the concentration range of 50–200 µM. In 769-p cells significant (P<0.05) growth suppression was also detected at a lower concentration (20 µM). Although of a small magnitude (~13%), we did detect a significant (P<0.05) growth stimulation in A-498 cells in response to a lower concentration (10 µM) of curcumin.

It has been reported that genetic or epigenetic variations in VHL can be detected in approximately 90% of human RCC cases. Given that 769-p cells do not express VHL mRNA, we used this VHL-deficient system for our subsequent studies that examined the mechanism by which curcumin suppressed ccRCC cell growth.

![Figure 1. Curcumin inhibits ccRCC cell proliferation and viability. 769-p cells (A), Caki-1 cells (B), 786-o cells (C), ACHN cells (D) and A-498 cells (E) were treated with the indicated concentrations of curcumin. After 3 days cell viability was detected using the Cell Titer-Glo Luminescent Cell Viability Assay. Data were calculated as percentage of vehicle control, and are the mean ± standard error of the mean, SEM of three independent experiments. Asterisks (*) designate a significant difference (P<0.05) from control values.](image)
We next examined the kinetics of curcumin-induced inhibition of 769-p cell proliferation. Our data showed that compared to vehicle treatment, 5 µM curcumin had no significant effect on 769-p cell proliferation at either the early time points or later time points (Figure 2A). On the other hand, 50 µM curcumin had a significant inhibitory effect on cell proliferation as early as 1 day, and this inhibitory effect persisted up to 3 days.

To confirm that the growth inhibition detected in the cell viability assays was due to decreased DNA synthesis, we examined the effect of curcumin on BrdU incorporation in 769-p cells. A significant (P<0.05) inhibition of cell proliferation was detected with 20 µM curcumin, and maximal suppression of cell proliferation was detected with 100–200 µM curcumin (Figure 2B).

Curcumin undergoes degradation in serum-depleted cell culture media, resulting in the formation of trans-6-(4′-hydroxy-3′-methoxyphenyl)-2,4-dioxo-5-hexenal, ferulic acid, feruloyl methane and vanillin. To address the question of whether the growth suppressive effects of curcumin were mediated by the degradation products of curcumin, 769-p cells were treated with the indicated concentration of ferulic acid and vanillin, and cell proliferation determined after 2 days. Our data showed that 50 µM ferulic acid had no significant growth suppression of 769-p cells (Figure 2C). Although 50 µM vanillin did suppress 769-p cell proliferation (<10%), it was approximately ninefold less effective than curcumin (50 µM) at suppressing growth of 769-p cells.

Curcumin Blocks Colony Formation Ability, Induces Apoptosis and Blocks Migration of ccRCC Cells

Next, we questioned whether curcumin had any effect on the colony formation ability of 769-p cells. 769-p cells were seeded in medium containing 10% FBS and allowed to adhere for 24 hours. The medium was then replaced with fresh medium containing 10% FBS together with the indicated concentration of curcumin, and colony formation was monitored over the ensuing 2 weeks. We observed that 769-p cells have a robust ability to form colonies and that this is abolished by curcumin in a concentration-dependent manner, with a substantial decrease in the number of 769-p cell colonies detected in presence of 5 µM curcumin (Figure 3A). At higher concentrations of curcumin (20–200 µM) the ability of 769-p cells to form colonies was completely ablated. Therefore, we questioned whether curcumin would induce apoptosis in ccRCC cells. To test this, 769-p cells were treated with the indicated concentrations of curcumin for 24 hours, washed, and incubated with JC-1 dye (a mitochondria-specific dual fluorescence probe) per manufacturer instructions (Cell Technologies).
In vehicle- and 5 μM curcumin-treated cells, 93.31% and 92.01% of the cells were detected with intact mitochondrial membranes, respectively. However, in 50 μM curcumin-treated cells, only 29.86% of cells presented intact mitochondrial membranes while 69.64% of the cell population presented with disruptions in the mitochondrial membrane (Figure 3B), indicating increased mitochondrial membrane depolarization.

To examine whether the increase in mitochondrial membrane depolarization would lead to increased cell death, 769-p cells were treated with the indicated concentrations of curcumin for 24 hours, and following trypsinization, annexin V-FITC binding and propidium iodide staining were analyzed by flow cytometry. Our results showed that in vehicle- and 5 μM curcumin-treated cells, 1.74% and 2.29% of cells, respectively, underwent apoptosis (Figure 3C). However, treatment with 50 μM curcumin resulted in 75.49% of cells undergoing cell death.

Since ccRCC tumors are highly metastatic, we questioned whether curcumin would block migration of ccRCC cells. To test this, 769-p cells were grown to monolayer confluence and a scratch wound was inflicted. Cells were incubated overnight in FBS-containing medium together with either vehicle or curcumin. Our data showed that in both vehicle- and 5 μM curcumin-treated cells, wound closure was complete (Figure 3D). However, 50 μM curcumin significantly inhibited the migration of 769-p cells, as evidenced by lack of wound closure.

**Curcumin Decreases Phosphorylation of NFκB and STAT3 in ccRCC Cells**

Given the important role of NFκB and STAT3 in ccRCC pathogenesis, and since curcumin has been shown to suppress NFκB and STAT3 transcriptional activity, we questioned whether the ability of curcumin to induce apoptosis in ccRCC cells is accompanied with decreased NFκB and STAT3 activation. Because transcriptional activity of NFκB and STAT3 is positively regulated by phosphorylation, the impact of curcumin on the phosphorylation status of p65 NFκB and STAT3 was examined by western blotting with antibodies that specifically detect either the phosphorylated or total form of p65 NFκB and STAT3. Our data demonstrated...
that curcumin significantly (P<0.05) decreased phosphorylation of p65 NFκB (S536) by 27.18% and 48.71% at concentrations of 20 and 50 µM, respectively (Figure 4A, 4B). In addition, 20 and 50 µM curcumin significantly (P<0.05) decreased STAT3 (S727) phosphorylation in 769-p cells by 31.14% and 48.76%, respectively (Figure 4C, 4D). The observed decrease in the phosphorylated forms of p65 NFκB and STAT3 were not due to an effect of curcumin on the expression levels of p65 NFκB and STAT3 (Figure 4A, 4C).

DISCUSSION
Both in the United States and worldwide, the incidence of ccRCC and ccRCC-related mortality has increased over the past decade. The National Cancer Institute reports that approximately 54,000 new cases of renal cancer are diagnosed and approximately 13,000 deaths by renal cancer occur annually. Although ccRCC is highly resistant to chemotherapy/radiotherapy, a better understanding of the biology of ccRCC has led to the identification of several signaling pathways currently being examined as potential targets. It is now well-established that constitutively active STAT3 and NFκB participate in ccRCC pathogenesis.9,14 Thus, suppression of STAT3 and NFκB provides a novel therapeutic approach to suppressing ccRCC cell proliferation. Given the lack of effective therapies for ccRCC, and the well-documented ability of the dietary polyphenol curcumin to suppress STAT3- and NFκB-mediated signaling, we examined whether ccRCC tumor suppression could be achieved with curcumin. It is also of interest to note that the age-adjusted incidence of kidney cancer in the United States is the highest in the world and approximately six times greater than rates in Asian countries where curcumin is regularly consumed through diet. Our in vitro study reveals that curcumin is effective at inducing apoptosis and suppressing migration of ccRCC cells.

In vitro characterization of the growth suppressive effects of curcumin in ccRCC cells revealed that curcumin, in a concentration- and time-dependent manner, suppressed 769-p cell viability and colony formation ability, induced apoptosis and blocked migration. We confirmed that the growth suppressive effect of curcumin was not cell type-specific, as in addition to the 769-p cells, curcumin-induced growth suppression was detected in a panel of human ccRCC cell lines, including Caki-1, 786-o, ACHN and A-498 cells. We also examined the ability of curcumin to suppress the growth of RENCA (mouse renal carcinoma cells) and observed a similar growth suppressive pattern (data not shown). Of particular significance is the ability of curcumin to suppress the growth of IFN-α–resistant 786-o cells.29 Our future studies will examine whether curcumin sensitizes IFN-α–resistant ccRCC cells to IFN-α–induced growth suppression.

We next questioned whether curcumin would decrease viability of normal renal proximal epithelial tubule cells. We observed that curcumin failed to induce apoptosis in cultured normal rat renal tubular epithelial cells (data not shown). Our observations are consistent with both in vitro and in vivo studies demonstrating a protective effect of curcumin on renal tubular epithelial cells.30,31 Taken together, our data clearly showed that curcumin could be an effective growth suppressor of ccRCC cells.

![Figure 4](image_url)

Figure 4. Curcumin decreases phosphorylation and transcriptional activity of NFκB and STAT3 in ccRCC cells. 769-p cells were treated with the indicated concentrations of curcumin for 30 minutes, and cell lysates were subjected to western blotting with anti-phospho p65 NFκB antibody (A, top) and anti-total p65 NFκB antibody (A, bottom), or anti-phospho STAT3 antibody (C, top) and anti-total STAT3 antibody (C, bottom). Data presented are from a single experiment and representative of three independent experiments. Western blots from all three experiments were quantified as described in the text. Fold change in expression of p-p65 NFκB (B) and p-STAT3 (D) from control (0 µM curcumin) was determined. Data are the mean (± SEM) of three independent experiments. Asterisks (*) designate a significant difference (P<0.05) from control values.
In most cases metastasis has already occurred when ccRCC is diagnosed. Therefore, the ability of curcumin to block cell migration of ccRCC cells was examined. Our wound healing assay data showed that curcumin was effective at blocking serum-induced wound closure.

Since degraded curcumin is known to yield dietary ferulates, especially in buffered medium and serum-free medium, we questioned whether the growth suppressive effects of curcumin we observed were due to the generation of ferulic acid and vanillin. However, because 50 µM ferulic acid had no significant growth suppressive effect on 769-p cell viability and vanillin’s modest growth suppressive effect on 769-p cell viability was approximately nine times less than curcumin at an equimolar concentration, we concluded that the growth suppressive effect of curcumin on ccRCC cell viability is not mediated by its degradation products, suggesting a direct effect of free curcumin. Our conclusion would be consistent with a response to a query raised from a recent article that described the antitumor effects of curcumin in pancreatic cancer patients. In their rebuttal the authors demonstrated that the suppressive effect of curcumin on tumor necrosis factor-α–activated NFκB in human myeloid cells is not mediated by dietary ferulates, as both ferulic acid and vanillin failed to block NFκB activation by this protein.

Phosphorylation of the p65 subunit of NFκB regulates the DNA binding and transcriptional activity of NFκB. Likewise, in melanocytic cells, constitutively phosphorylated STAT3 has been shown to accumulate in the nucleus and promote cell survival. In examining the impact of curcumin on NFκB- and STAT3-mediated transcription in ccRCC cells, we found there is a robust expression of constitutively phosphorylated p65 NFκB and STAT3 in 769-p cells. Further, when 769-p cells are exposed to curcumin, a rapid decrease in the phosphorylation of transcription factors p65 NFκB and STAT3 was observed. Although our data clearly showed that curcumin decreases STAT3 and NFκB phosphorylation, the mechanism of this suppression in ccRCC cells remains to be determined. One of the key kinases that phosphorylate p65 NFκB is IKKα, which phosphorylates p65 NFκB on serine 536. In multiple myeloma cells, curcumin inhibited constitutively activated IKK. It is possible the observed decrease in p65 NFκB phosphorylation in ccRCC cells could be due to curcumin-induced suppression of IKKα activity. Likewise, the inhibitory effect of curcumin on STAT3 phosphorylation could involve suppressing receptor-associated Janus kinase(s) or the nonreceptor tyrosine kinase Src. Interestingly, Yonezawa et al. demonstrated the suppression of RCC cell growth by Src kinase inhibitor PP1 and that this was accompanied with decreased STAT3 activation. Our future studies will examine the role of IKKα and Src in mediating the growth suppressive effects of curcumin in ccRCC cells.

Recent studies suggested the potential of targeting NFκB and STAT3 signaling axes in ccRCC as novel therapeutic approaches. To this end, the proteasome inhibitor bortezomib induced apoptosis in ccRCC cells in a NFκB-dependent manner; however, just the suppression of NFκB was not sufficient to induce apoptosis in ccRCC cells, demonstrating the requirement of engaging additional signaling targets in inducing apoptosis in ccRCC cells. It is reasonable to conclude that the ability of curcumin to target two essential signaling arms in ccRCC, STAT3 and NFκB, might be the underlying basis of its pro-apoptotic effect.

Although curcumin is an effective and selective (i.e. targets tumor cells but not normal cells) antitumor agent, its use in a clinical setting has been limited, primarily due to its well-documented poor pharmacokinetic profile. To overcome this limitation, novel approaches to enhancing the bioavailability of curcumin are now the focus of several studies. These include coadministration of curcumin and piperine (an inhibitor of glucuronidation), which resulted in a 2,000% increase in bioavailability of curcumin. Another approach has led to the generation of nanoparticles of curcumin. Nanoparticle curcumin was effective at suppressing the growth of MCF-7 breast cancer cells, and at inhibiting NFκB activity and inducing apoptosis in human pancreatic tumor cell lines. Liposomal curcumin also was shown to be an effective anticancer agent in a pancreatic cancer xenograft model. Additionally, complexing curcumin with micelles or phospholipids has proved to improve its bioavailability. Finally, synthetic curcumin analogs (e.g. EF-24, HO-3867) were reported to have increased bioavailability when compared to curcumin and are more effective than curcumin at suppressing tumor growth. Interestingly, following an intraperitoneal administration of HO-3867 in rats, the synthetic curcumin analog was detected in the blood, liver, stomach and kidney.
Given the excellent safety profile of curcumin, and the recent efforts aimed at enhancing its bioavailability, we anticipate that clinical trials examining the efficacy of curcumin or its synthetic analogs in suppressing ccRCC tumorigenesis will be conducted.

CONCLUSIONS
We report a robust growth suppressive and pro-apoptotic in vitro effect of curcumin on ccRCC cells. Although in vitro findings do not necessarily translate into clinical care, if the growth suppressive effects of curcumin on ccRCC are validated in clinical trials, this therapeutic approach could complement existing therapies.

Patient-Friendly Recap
• Curcumin, a commonly used food additive in Southeast Asia, is known to inhibit the growth of several types of cancer cells in experimental settings.
• The authors studied whether curcumin could block growth and migration of malignant kidney cancer cells.
• The authors found that curcumin decreased the viability of kidney cancer cells as well as their ability to migrate.
• Given its few side effects, curcumin (or its synthetic analogs) could be considered for clinical trials aimed at developing novel therapies for kidney cancer.

Conflicts of Interest
None.

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