

## Curcumin Treatment Suppresses IKK $\beta$ Kinase Activity of Salivary Cells of Patients with Head and Neck Cancer: A Pilot Study

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### Abstract

**Purpose:** To determine whether curcumin would inhibit I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ) kinase activity and suppress expression of proinflammatory cytokines in head and neck squamous cell carcinoma cancer (HNSCC) patients.

**Experimental Design:** Saliva was collected before and after subjects chewed curcumin tablets. Protein was extracted and IKK $\beta$  kinase activity measured. Interleukin (IL)-6 and IL-8 levels in the salivary supernatants were measured by ELISA. IL-6, IL-8, and other interleukin were also measured independently with ELISA to confirm the inhibitory effect of curcumin on expression and secretion of salivary cytokines.

**Results:** Curcumin treatment led to a reduction in IKK $\beta$  kinase activity in the salivary cells of HNSCC patients ( $P < 0.05$ ). Treatment of UM-SCC1 cells with curcumin as well as with post-curcumin salivary supernatant showed a reduction of IKK $\beta$  kinase activity. Significant reduction of IL-8 levels ( $P < 0.05$ ) was seen in post-curcumin samples from patients with dental caries. Although there was reduced IL-8 expression in 8 of 21 post-curcumin samples of HNSCC patients, the data did not reach statistical significance. Saliva samples from HNSCC patients were also analyzed in a blinded fashion for expression of cytokines. IL-10, IFN- $\gamma$ , IL-12p70, and IL-2 clustered together, and granulocyte macrophage colony stimulating factor and TNF- $\alpha$  clustered together. Log<sub>10</sub> ratio analysis showed decrease in expression of all nine cytokines in both the salivary supernatant and salivary cells of curcumin-treated samples.

**Conclusions:** Curcumin inhibited IKK $\beta$  kinase activity in the saliva of HNSCC patients, and this inhibition correlated with reduced expression of a number of cytokines. IKK $\beta$  kinase could be a useful biomarker for detecting the effect of curcumin in head and neck cancer. *Clin Cancer Res*; 17(18); 5953–61. ©2011 AACR.

### Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most morbid of human cancers, and affects 600,000 people worldwide annually, including 42,000 people in the United States (1, 2). HNSCC includes oral,

laryngeal, and pharyngeal malignancies, with about 40% of these arising in the oral cavity (1). Over the years, there have been many changes in the way HNSCC is treated. Current treatment methods for advanced head and neck cancer include radiation therapy, chemotherapy, and surgery. Despite medical advancements, the overall survival rates for patients with advanced HNSCC have remained poor. The 5-year survival rate for all patients with head and neck cancers is 57%, and for patients with stage III and IV oral cancers the rates are even lower at 10% to 20% (1, 3). In addition, 30% to 50% of patients develop local or regional recurrence, and another 10% to 40% of patients develop second primary tumors of the aerodigestive tract due to field cancerization (4). As a result, there have been ongoing investigations into alternative therapies with reduced morbidity as well as reduced toxicity to minimize the adverse effect on cancer patients.

Curcumin (diferuloylmethane), the yellow pigment that is derived from the East Indian plant *Curcuma longa*, is the major antioxidant and anti-inflammatory substance found in turmeric. For centuries, turmeric has been used as a spice

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

Curcumin shows promise as a potential adjuvant treatment for patients with head and neck squamous cell carcinoma. Its suppressive activity seems to involve inhibition of I $\kappa$ B kinase (IKK) kinase activity, preventing NF- $\kappa$ B translocation to the nucleus. *In vitro* studies have showed that curcumin reduces the expression of inflammatory cytokines through the inhibition of IKK $\beta$  kinase activity. In this study, we show that curcumin inhibits IKK $\beta$  kinase activity in the saliva of head and neck cancer patients, and this inhibition correlates with a reduced expression of a number of cytokines. Our data suggest that IKK $\beta$  kinase could be a useful biomarker for detecting the effect of curcumin in head and neck cancer. Our findings also support investigation of a larger set of head and neck cancer patients to determine the clinical utility of curcumin in the suppression of IKK $\beta$  kinase activity and the inhibitory effect on the inflammatory cytokines.

and food-coloring agent and has served as a naturally occurring medicine to treat inflammatory disorders (5, 6). Preclinical studies have consistently shown that curcumin inhibits cancer proliferation in various cancer cell lines, including breast, cervical, and pancreatic cancers (6–8). One study by Hanif and colleagues (7) tested curcumin's effect on colon adenocarcinoma cell lines and saw as much as 96% reduction in the number of colon cancer cells following curcumin administration. Numerous other studies have concluded that curcumin can act as a chemopreventive agent both *in vitro* and *in vivo* in animal models. Limtrakul and colleagues (9) showed that dietary curcumin administration significantly inhibited the development of skin tumors and also reduced skin tumor volume in mice. Our laboratory has also found that curcumin suppresses cancer cell proliferation of head and neck cancer cell lines *in vitro* and *in vivo* in nude mice with xenograft tumors (10–12). Furthermore, we have found curcumin to be nontoxic in the organs of the mice. Even though various mechanisms by which curcumin acts are not completely understood, studies have shown that curcumin downregulates NF- $\kappa$ B, an inducible transcription factor that is involved in the activation of a number of cell processes, including cell growth, invasion, and metastasis (5, 12, 13).

NF- $\kappa$ B functions in a variety of human diseases, such as asthma, AIDS, septic shock, and cancer. This factor is activated in many cell types in response to a broad range of stimuli, which include mitogens, inflammatory cytokines, and extracellular stress, such as cigarette smoke and UV irradiation (14, 15). NF- $\kappa$ B activation occurs as it is transported from the cytoplasm to the nucleus upon phosphorylation/degradation of its inhibitory molecule I $\kappa$ B (15). The nuclear translocation leads to DNA binding and transcription of growth factors and cytokines (VEGF,

TNF, MCP-1, Bcl-2, COX-2, and cyclin D1). I $\kappa$ B kinase (IKK) kinase, a complex consisting of 3 proteins: IKK $\alpha$ , IKK $\beta$ , and IKK $\gamma$  [also known as the NF- $\kappa$ B essential modulator (NEMO)], is responsible for the phosphorylation of the I $\kappa$ B $\alpha$  subunit of I $\kappa$ B, resulting in ubiquitination and rapid degradation (15). Previously, we have shown that curcumin inhibits IKK kinase activity, thereby preventing NF- $\kappa$ B translocation to the nucleus (11). Recently, our laboratory has also shown that curcumin binds to IKK $\beta$  protein to exert the inhibitory effect (16). Thus, curcumin's modulation of the IKK kinase activity of the NF- $\kappa$ B pathway leads to decreased NF- $\kappa$ B transcription activation and reduced cell growth.

Higher concentrations of proinflammatory and proangiogenic cytokines are correlated with advanced stage, metastatic disease, or large tumor burdens of various cancers (17–19). One study (17) identified elevated levels of the cytokines interleukin (IL)-6, IL-8, and VEGF in patients with HNSCC compared with patients with laryngeal papilloma or age-matched controls. Another study (18) detected increased concentrations of IL-8 in the saliva of patients with oral cavity and oropharyngeal squamous cell carcinoma compared with age- and sex-matched control subjects. Our laboratory has found that the level of IL-6 and IL-8 expression correlated with the aggressiveness of the HNSCC cell lines, and there was dose-dependent inhibition of IL-6 and IL-8 expression with curcumin treatment (11). Because the *in vitro* studies have shown that curcumin reduced the expression of inflammatory cytokines through the inhibition of IKK $\beta$  kinase activity, we wanted to determine whether curcumin would also inhibit IKK $\beta$  kinase activity and the expression of IL-6 and IL-8 *in vivo* when administered to patients with HNSCC.

### Materials and Methods

#### Cell lines

The UM-SCC1 (oral cancer cell line) was grown in minimum essential medium containing Earle's salts, L-glutamine, 10% FBS, and 1% nonessential amino acids.

#### Patient selection

Patients were recruited from the Division of Head and Neck Surgery and the Dental Clinic associated with the Veterans Affairs Greater Los Angeles Healthcare System with informed consent under Institutional Review Board–approved protocols over an 8-month period. The sample group consisted of 34 subjects (13 with dental caries and 21 with head and neck cancer; Table 1). The control group included 5 disease-free individuals. There were a total of 39 subjects in this study (13 with dental caries, 21 with head and neck cancer, and 5 healthy volunteers). The group with dental caries ranged in age from 42 to 60 years. The group with head and neck cancer was in age range of 36 to 90 years. The group of healthy volunteers ranged in age from 51 to 91 years. All subjects in all groups were male.

**Table 1.** Oral cancer subject information

| Sample no. | Site           | Stage  | Differentiation       |
|------------|----------------|--|-----------------------|
| 2          | Tongue         | No prior   | Moderate              |
| 4          | Tongue         | T <sub>2</sub> N <sub>2</sub> BOT, recurrent, s/p chemo XRT        | Poor                  |
| 5          | Oral           | T <sub>4</sub> N <sub>2</sub> M <sub>1</sub> —no prior             | Moderate              |
| 6          | Oral           | T <sub>2</sub> N <sub>2B</sub> —no prior                           | Poor                  |
| 7          | Oral           | T <sub>4</sub> N <sub>3</sub> —no prior                            | Moderate              |
| 8          | Oral           | T <sub>3</sub> N <sub>0</sub> recurrent, s/p XRT, path only by FNA |                       |
| 9          | Pharynx        | T <sub>4</sub> N <sub>2B</sub> —no prior                           | Poor                  |
| 10         | Oral           | T <sub>4</sub> N <sub>0</sub> —prior XRT                           | No note               |
| 11         | Base of tongue | T <sub>4A</sub> N <sub>2C</sub> no prior treatment                 | Only FNA              |
| 12         | Floor of mouth | T <sub>2</sub> N <sub>1</sub>                                      | Basaloid <sup>a</sup> |
| 13         | Oral           | T <sub>2</sub> N <sub>3</sub> —no prior                            | Undifferentiated/Poor |
| 14         | Tonsil         | T <sub>4</sub> N <sub>0</sub> —no prior                            | Poor                  |
| 15         | Tonsil         | T <sub>2</sub> N <sub>1</sub> —no prior                            | Moderate              |
| 16         | Tonsil         | T <sub>2</sub> N <sub>1</sub>                                      | Basaloid <sup>a</sup> |
| 17         | Tonsil         | Recurrent—previous surgery only (no XRT, chemo)                    | Moderate              |
| 18         | Oral           | T <sub>2</sub> N <sub>1</sub> —no prior                            | Poor                  |
| 19         | Oral           | T <sub>4</sub> N <sub>2C</sub> —no prior                           | Poor                  |
| 20         | Oral           | T <sub>2</sub> N <sub>2C</sub> —no prior                           | No note, only FNA     |
| 21         | Tongue         | T <sub>4</sub> N <sub>0</sub> —no prior                            | Moderate              |
| 22         | Tongue         | T <sub>1</sub> N <sub>0</sub> —recurrent                           | Moderate              |
| 23         | Tongue         | T <sub>1</sub> N <sub>0</sub> —no prior treatment                  | Moderate              |

Abbreviations: FNA, fine needle aspiration; XRT, X-ray therapy.

<sup>a</sup>Basaloid is very aggressive type.

### Saliva collection

Saliva (5 mL) was collected in 50-mL tubes, without any PBS or media, before and 1 hour after subjects chewed 2 curcumin caplets (Jarrow Formulas Curcumin 95) for 5 minutes. The total curcumin concentration of the chewed caplets was 1,000 mg. All subjects were required to refrain from eating, drinking, smoking, or using oral hygiene products throughout the duration of the experiment. The saliva samples were immediately placed on ice, centrifuged at 3,500 rpm for 15 minutes at 4°C. The supernatant was removed from the cell pellets and stored in 1 mL aliquots. Both the cell pellets and the supernatants were stored at –80°C.

### Treatment of UM-SCC1 cells with salivary samples

The UM-SCC1 cell line was plated in 100-mm tissue culture dishes and allowed to grow in 10 mL medium for 24 hours to reach 80% confluency. The cells were serum starved for 24 hours and preincubated with TNF- $\alpha$  (10 ng/mL) for 15 minutes at 37°C. The cells were then treated with 2 mL serum-containing media or with 1 mL of salivary supernatant and 1 mL of medium and incubated at 37°C for 4 hours. Cell extracts were prepared in kinase buffer [50 mmol/L Tris-hydrochloride (pH 7.5), 20 mmol/L MgCl<sub>2</sub>, 0.20 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 10 mmol/L  $\beta$ -glycerophosphate, 4 mmol/L diethiothreitol], and IKK $\beta$  activity was measured by the IKK $\beta$  Kinase Assay Kit (Cell Signaling Technology). Cells treated with dimethyl sulf-

oxide (DMSO) alone or curcumin dissolved in DMSO were used as controls.

### IKK $\beta$ kinase activity assay

IKK $\beta$  kinase activity was measured by using the protocol provided with the assay kit (HTScan IKK $\beta$  Kinase Assay Kit; Cell Signaling Technology). Proteins isolated from the salivary cells or from the UM-SCC1 cells were mixed with the IKK $\beta$ -specific substrate, biotinylated I $\kappa$ B $\alpha$  [phosphorylated at serine residue 32 (ser 32)] peptide (3  $\mu$ mol/L), and adenosine triphosphate (400  $\mu$ mol/L) in a 50- $\mu$ L reaction mixture. The assay mixture was incubated at room temperature for 30 minutes followed by the addition of the stop buffer (50 mmol/L EDTA). The reaction mixture was then transferred to a 96-well streptavidin-coated plate (Delfia Streptavidin-coated yellow plate, 96-well plates; PerkinElmer Inc.) and incubated at room temperature for 1 hour. The plate was washed 3 times with polysorbate 20-containing PBS and the phosphorylated I $\kappa$ B $\alpha$  (ser 32) rabbit monoclonal antibody (Cell Signaling Technology) was added. The reaction was then incubated for 2 hours at room temperature and washed 3 times with the PBS solution with 0.02% of Tween-20 (PBST). Then the anti-rabbit horseradish peroxidase secondary antibody was added and the plate was incubated for an additional 30 minutes at room temperature. After 5 more washes with PBST, the 3,3',5,5'-tetramethylbenzidine substrate (Cell Signaling Technology) was added. Finally, the plate was incubated

for 15 minutes at room temperature, and the reaction was stopped by using the stop solution. Absorbance of the colored reaction product was measured at 450 nm by using a 96-well microplate reader (PowerWave XS; BioTek Instruments, Inc.). The study was carried out thrice and each time in triplicates.

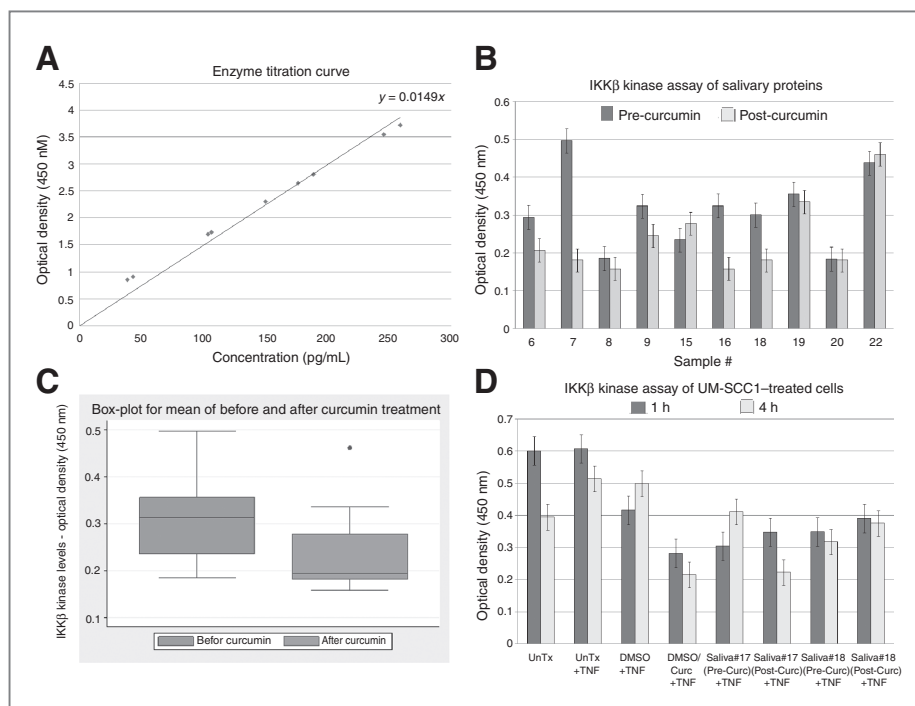
### Quantification of cytokine levels in salivary supernatants

We initially measured IL-6 and IL-8 concentrations by using an ELISA kit according to the manufacturer's protocol (R&D Systems). The supernatant of each saliva sample (100  $\mu$ L) was tested in triplicate. After development of the colorimetric reaction, the absorbance at 450 nm was measured by using a 96-well microplate reader (PowerWave XS; BioTek Instruments, Inc.). The absorbance readings were then converted to picograms per milliliter on the basis of standard curves obtained with the recombinant cytokine. The lower limits of sensitivity for the IL-6 and IL-8 assays were 3.12 and 31.2 pg/mL, respectively. The ELISA studies were repeated once.

Cytokine levels [granulocyte macrophage colony stimulating factor (GM-CSF), TNF $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-10, IL-12p70, IL-2, IL-6, and IL-8] in saliva and solubilized saliva pellets were determined by using the Human 9-Plex Ultra-sensitive Electrochemiluminescent MULTI-SPOT Assay from Meso Scale Discovery. Saliva pellet proteins were solubilized through the addition of 100 to 200  $\mu$ L of a detergent-containing extraction buffer. Twenty-five microliters of saliva or saliva pellet protein were used for the MULTI-SPOT assays which were carried out according to the manufacturer's directions. Cytokines were detected by the SECTOR Imager 6000 CCD camera (Meso Scale Discovery).

### Statistical evaluation

Student's *t* test and the Wilcoxon signed-rank test were carried out to determine the significance of IKK $\beta$  enzyme activity changes after curcumin treatment in salivary cell samples. Two-sided 2-sample *t* test was carried out to determine the significance of IL-6 and IL-8 expression level changes in curcumin-treated versus untreated salivary



**Figure 1.** Curcumin inhibits salivary cell IKK $\beta$  kinase activity of head and neck cancer patients. A, dose-dependent curve of IKK $\beta$  kinase activity was achieved by using 5 IKK $\beta$  protein concentrations in increments of 50 pg/mL. The enzyme activity is linear up to 250 pg/mL tested in the present assay. B, protein was extracted from salivary samples collected from patients with HNSCC before and after a 1-hour curcumin treatment. IKK $\beta$  kinase activity was measured by the IKK $\beta$  Kinase Assay Kit. Six of 10 samples show a decrease in IKK $\beta$  kinase activity, with 3 samples (#7, 16, and 18) indicating a 50% reduction post-curcumin treatment. C, box plot showing expression level of IKK $\beta$  enzyme activity in pre- and post-curcumin salivary samples. The results indicate a statistically significant inhibition of IKK $\beta$  kinase activity by curcumin with  $P < 0.05$ . D, the UM-SCC1 cell line was treated with DMSO, curcumin dissolved in DMSO, or saliva samples collected pre- and post-curcumin treatment. The cells were also pretreated with TNF- $\alpha$  for 15 minutes. As reported earlier (11), addition of curcumin inhibits IKK $\beta$  kinase activity of UM-SCC1 cells. One of the 2 post-curcumin salivary samples (sample 17) exhibits an inhibitory effect on IKK $\beta$  kinase activity, indicating usefulness of this methodology to determine IKK $\beta$  kinase levels in the saliva of HNSCC patients. The IKK $\beta$  kinase assay carried out at 4 hours in untreated samples (UnTx) shows a reduction in comparison with 1 hour due to loss of growth factors. The 1-hour treatment of UN-SCC1 cells with pre-curcumin saliva/medium (50%:50%) shows reduced IKK $\beta$  kinase activity in comparison with untreated controls due to the presence of half the level of growth factors in this mixed medium.

supernatant samples. Hierarchical clustering was employed by using Genepattern (Broad Institute) to identify patient and cytokine expression clusters in both the saliva pellet and supernatant samples. We centered the rows and columns to their respective median values and used the Euclidean distance measure. Clustering was carried out separately on pre-curcumin treatment data, post-curcumin treatment data, and on the effect of curcumin on cytokine expression levels (using the log of the ratio between post- and pre-curcumin levels).

## Results

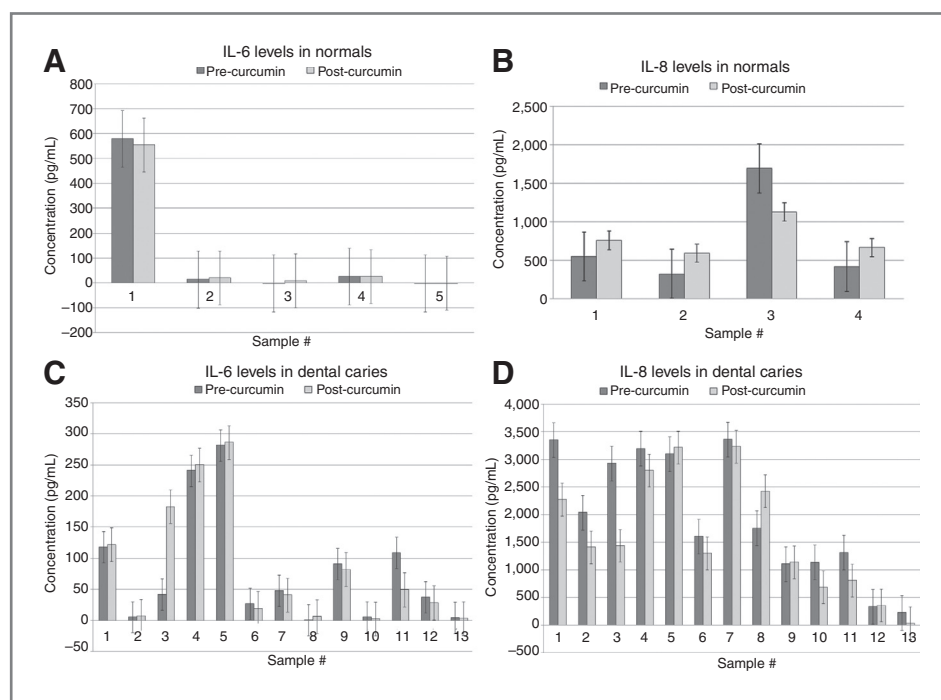
### Inhibition of IKK $\beta$ kinase activity by curcumin in the salivary cells of HNSCC patients

An enzyme titration for IKK $\beta$  kinase activity was carried out by the IKK $\beta$  Kinase Assay Kit to derive a standard curve that could be used for the protein measurements in salivary samples. The enzyme activity was linear up to 250 pg/mL protein concentration tested in this investigation (Fig. 1A). Pretreatment saliva samples had IKK $\beta$  concentrations ranging from 12 to 33.6 pg/mL, whereas posttreatment saliva samples had IKK $\beta$  concentrations between 10.6 and 31 pg/mL (Fig. 1B). We found 6 of 10 samples (60%) to have a reduction in IKK $\beta$  kinase activity following 1 hour of curcumin treatment, with 3 of the samples showing a

50% reduction. Statistical significance ( $P < 0.05$ ) was observed for the inhibitory effect of curcumin on the IKK $\beta$  kinase activity (Fig. 1C). Two samples did not show a change and the remaining 2 samples had less than a 10% increase in enzyme activity post-curcumin treatment. These results corroborated our hypothesis that curcumin inhibits IKK $\beta$  kinase activity in the salivary cells of patients with head and neck cancer.

### Inhibition of IKK $\beta$ kinase activity in curcumin-treated saliva samples by the *in vitro* assay

To determine whether curcumin has IKK $\beta$  kinase inhibitory activity on growing tumor cells, the salivary supernatants were used to treat the UM-SCC1 in an *in vitro* cell assay. TNF- $\alpha$  was also added to increase the enzyme activity. Two samples for which we had sufficient salivary supernatant were tested in the *in vitro* assay. As reported earlier (10), we used DMSO and curcumin dissolved in DMSO as controls. A 4-hour treatment with curcumin showed a 60% reduction in enzyme activity (Fig. 1D). Treatment of the UM-SCC1 cells with one of the post-curcumin salivary samples (#17) resulted in a 50% reduction in enzyme activity (Fig. 1D) reflecting a possible reduction in proinflammatory cytokine concentration in the supernatant sample. It is also possible that the enzyme inhibition was due to curcumin still present



**Figure 2.** Inhibitory effect of curcumin on IL-8 expression in the saliva of patients with dental caries. A, the IL-6 expression is less than 5 pg/mL in 4 of 5 normal individuals, which is similar to the expression level reported in the literature. Individual 1 has high level expression that could reflect an inflammatory condition. B, IL-8 expression in 3 individuals resembles the expression range reported in the literature. Individual 3 has 3 times the expected expression. Appreciable changes were not observed in IL-6 or IL-8 levels after curcumin treatment. C, of the 13 patients with dental caries tested, one sample (#11) shows a 50% reduction and another sample (#3) has a 4-fold increased expression of IL-6. Appreciable changes are not seen in other samples. D, 8 of the dental caries patients show reduced IL-8 expression and 4 of them show greater than 25% reduction (#1, 3, 11, and 13). One sample (#8) shows a 20% increase in IL-8 expression post-curcumin treatment.

in the saliva of this patient. Although inhibition was not observed with sample 18, the salivary cells of this sample have revealed an inhibitory effect on the enzyme activity (see Fig. 1B). It is likely then that a longer oral curcumin treatment might be required to induce a reduction in the level of cytokines released from some of the HNSCC patients.

### Curcumin treatment decreased IL-8 levels in saliva of patients with dental caries

To show further that the inhibitory effect can be seen in cytokines secreted in the saliva, we measured the IL-6 and IL-8 levels in the salivary supernatant of the head and neck cancer patients. As a control, we studied IL-6 and IL-8 levels in normal individuals and individuals with an inflammatory condition, dental caries. The IL-6 level in 4 of 5 normal individuals was less than 5 pg/mL, and the expression did not change after curcumin treatment (Fig. 2A). One sample had a 600 pg/mL concentration that did not vary after curcumin treatment. The IL-8 expression level in 4 individuals ranged from 300 to 1,700 pg/mL (Fig. 2B). Curcumin treatment resulted in a 30% decrease in 1 individual and appreciable changes were not seen in the other 3 samples.

The IL-6 and IL-8 expression in many of the samples collected from patients with dental caries were higher than the normal range. The IL-6 level was less than 50 pg/mL in 8 samples, less than 100 pg/mL in 3 samples, and more than 200 pg/mL in 2 samples (Fig. 2C). Curcumin treatment showed a 50% decrease in one sample (#11) and a 4-fold increase in another sample (#3; Fig. 2C). The IL-8 expression was less than 1,000 pg/mL in 5 samples, close to 2,000 pg/mL in 3 samples, and more than 3,000 pg/mL in 5 other samples (Fig. 2D). Thus, the IL-8 expression in 11 samples was higher than the level

observed for the normal individuals reflecting an inflammatory response to dental caries. Curcumin treatment resulted in decreased expression in 8 samples (62%) with greater than a 25% reduced expression in 4 samples (#1, 3, 11, and 13; Fig. 2D). One sample (#8) had a 20% increased expression and the remaining 4 samples did not show a change in expression. Statistical evaluation of the cytokine levels showed that the decrease in IL-8 expression in post-curcumin samples was significant ( $P < 0.05$ ).

### Modest inhibitory effect on IL-8 expression by curcumin in patients with head and neck cancer

The IL-6 and IL-8 levels in patients with head and neck cancer were greater than the levels observed in normal individuals. Expression levels were in the range of 4 to 700 pg/mL for IL-6 and 30 to 5,000 pg/mL for IL-8 (Fig. 3). Post-curcumin treatment, greater than a 25% decrease in IL-6 expression was seen in 2 samples (#10 and 20; 9%) and a 25% increased expression was noticed in 3 samples (#11, 13 and 22; 14%; Fig. 3A). Five samples (#2, 10, 16, 17, and 19) had a 20% reduction in IL-8 expression (24%) and 2 samples (#11 and 22) had greater than a 20% increased expression (9%; Fig. 3B). The remaining samples did not show an appreciable change, indicating a modest inhibitory effect on IL-8 post-curcumin treatment in these individuals. On the basis of these findings, we feel that curcumin may need to be administered for a longer time period in order for it to have a stronger inhibitory effect on the IL-6 and IL-8 levels in the saliva of head and neck cancer patients. It is also likely that higher curcumin concentrations coupled with an extended treatment time period may be required for the curcumin effect to be noticeable in HNSCC patients. Further studies are needed to confirm this phenomenon.

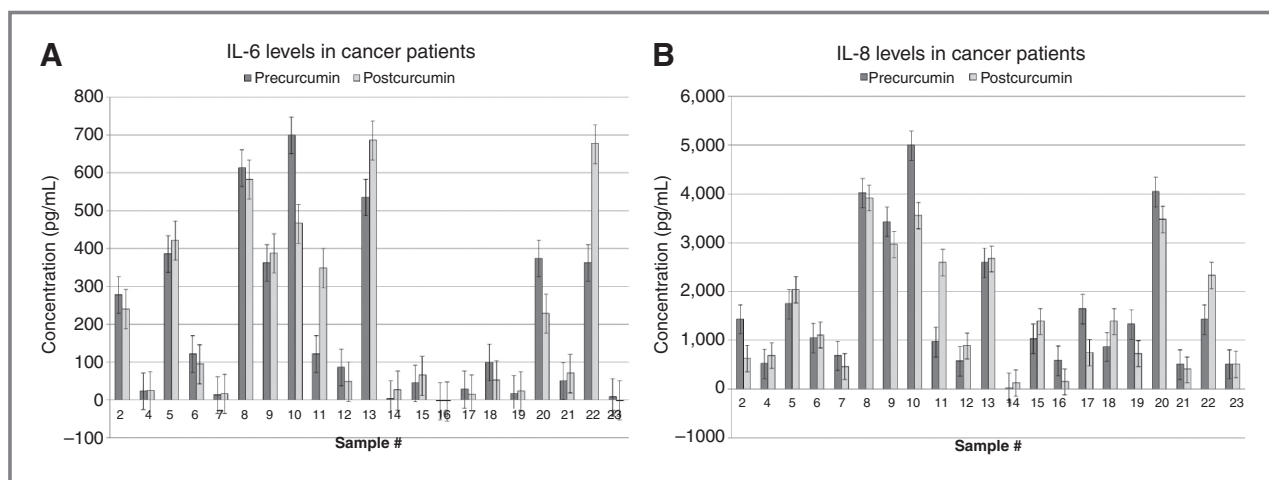


Figure 3. Modest inhibitory effect of curcumin on salivary IL-8 expression in head and neck cancer patients. A, of the 21 head and neck cancer patient samples analyzed, 2 samples (#10 and 20) show decreased expression and 3 samples (#11, 13, and 22) show increased expression of IL-6 following curcumin treatment. B, curcumin treatment results in greater than a 20% decreased expression of IL-8 in 5 samples (#2, 10, 16, 17, and 19). More than 20% expression of IL-8 is seen in 2 samples (#11 and 22) the same samples that have shown increased IL-6 expression after curcumin treatment. The modest inhibitory effect on IL-8 suggests that higher dose of curcumin and longer treatment time periods may be required to induce significant inhibitory effect on cytokine expression in the saliva of different head and neck cancer patients.

### Independent verification of the inhibitory effect of curcumin on cytokine expression in head and neck cancer

As an independent verification of the effect of curcumin on IL-6 and IL-8, and to assess whether curcumin regulates inflammatory response, we measured cytokine levels by using mesoscale discovery platform in 14 of the salivary samples. These studies were carried out in a blinded fashion in the Institute for Molecular Medicine, Uniformed Services University of Health Sciences School of Medicine, Bethesda, MD. Both the supernatant and cell pellet samples were analyzed. Clustering analysis and statistical significance were determined for the tumor site and recurrence. Clustering of the oral cancers separate from the tongue and tonsil were noticed (Supplementary Fig. S1). We could further observe clustering of IL-10, IFN- $\gamma$ , IL-12p70, and IL-2 as a group, and that of GM-CSF and TNF- $\alpha$  as another group in the cluster analysis. We also observed a statistically significant reduction in IL-10, IFN- $\gamma$ , and IL-12p70 expression in the salivary cells of the recurrent tumors in comparison with

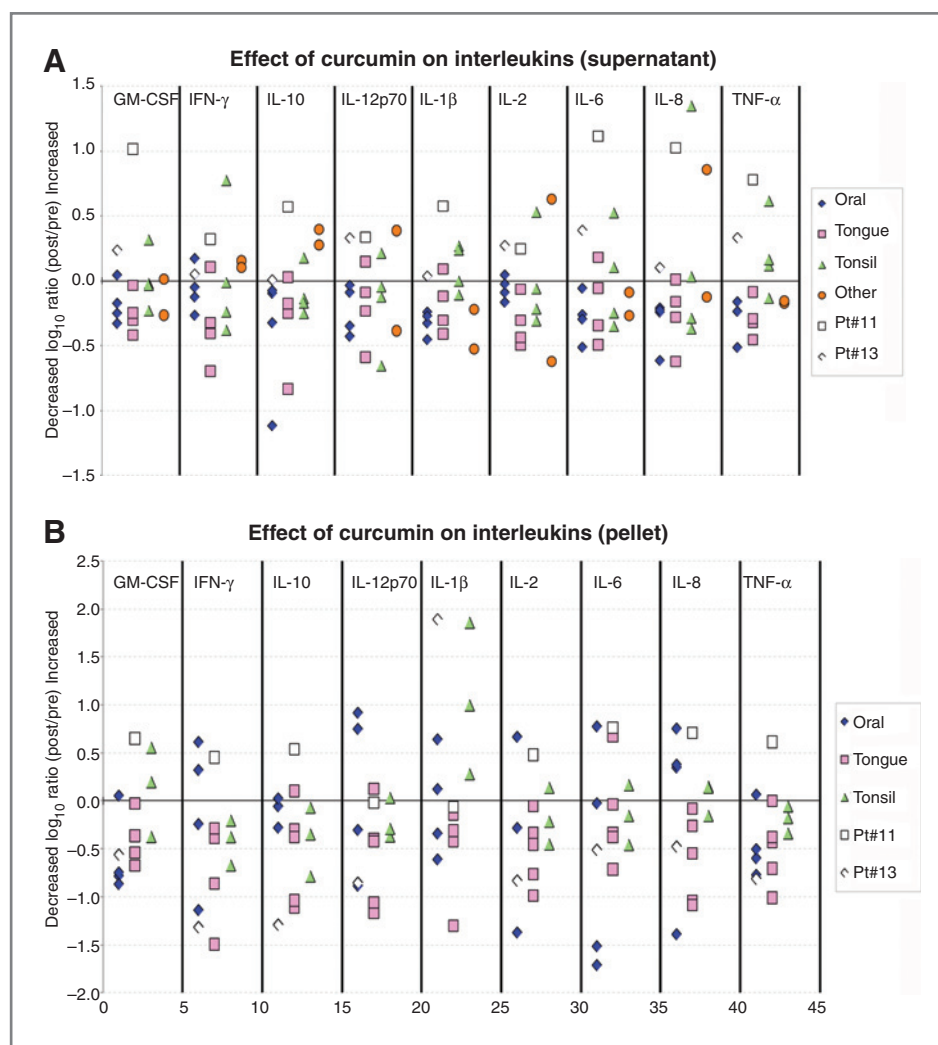
tumors without a prior tumor history (Supplementary Fig. S2). Finally, in a majority of samples, post- versus pre-curcumin log<sub>10</sub> ratio analysis showed a clear reduction in the expression (8–10 of 14 samples, 57%–71%) of all 9 cytokines in the salivary supernatant as well as in cell pellets (Fig. 4). The inhibitory effect was highly noticeable in tongue tumors.

### Discussion

HNSCC encompasses a wide range of tumors, including the nasopharynx, oral cavity, oropharynx, hypopharynx, and/or larynx. It is the fifth most common cancer in the world with one of the lowest 5-year survival rates (1). Current treatment modalities for HNSCC include surgery, radiation, and chemotherapy; however, these often result in significant morbidity especially for patients with late-stage HNSCC.

Curcumin, a food derivative that is mostly used as a spice and food coloring agent, has been found to possess

**Figure 4.** Inhibitory effect of curcumin on inflammatory cytokines. Analysis of 14 salivary samples using the Human 9-Plex Ultrasensitive Electrochemiluminescent MULTI-SPOT Assay system followed by the cluster analysis shows decreased expression of all 9 cytokines in supernatant (A) and in cellular pellet (B). Although the inhibition is variable as was seen in Figure 3, cluster analysis points to enhanced inhibitory activity on tongue tumors indicating the usefulness of curcumin in the treatment of this tumor type. Overexpression of IL-6 and IL-8 observed in the supernatant samples 11 and 13 confirmed the results obtained with the individual cytokine ELISA method (Fig. 3), indicating the usefulness of either of the methods to detect interleukin expression in salivary samples.



potent chemopreventive properties (5–8). Many studies have shown that curcumin has antiproliferative and proapoptotic effects against various tumors *in vitro* as well as *in vivo*. In addition, curcumin has been proven to be pharmacologically safe. To date clinical trials have not identified a maximum tolerated dose of curcumin in humans (8). Clinical studies have been administering up to 8,000 mg/d of curcumin to patients and have concluded that curcumin is nontoxic and poses minimal adverse effects on humans. One of the 8 pancreatic cancer patients receiving the oral curcumin showed remission, indicating the usefulness of curcumin in some cancer patients (20).

Inflammatory response from cancer is well documented and increased expression of proinflammatory cytokines have been observed in serum and saliva of cancer patients. St. John and colleagues (18) and Rhodus and colleagues (19) have shown statistically significant higher concentrations of IL-8 in the saliva of oral cancer patients in comparison with age- and sex-matched noncancer individuals. Secretion of cytokines such as IL-6, IL-8, and VEGF are related to increased activation of transcription factor NF- $\kappa$ B in the cancer patients. Therefore, it is hypothesized that the inhibition of NF- $\kappa$ B could play an important role in the control of cancer development.

We and others have documented constitutive activation of NF- $\kappa$ B in head and neck cancer cell lines (5, 10–12). We have also shown that HNSCC cell lines express IKK $\beta$  in the nucleus where the enzyme may be involved in the transcription activation of NF- $\kappa$ B (16). Cell line studies from our laboratory have shown that curcumin inhibits NF- $\kappa$ B activity through the inhibition of IKK $\beta$  kinase. In the present investigation, we have showed that curcumin downregulates IKK $\beta$  kinase activity of the salivary cells leading to the inhibition of cytokine expression in some cancer patients. The inhibitory effect of curcumin was highly noticeable in recurrent tumors and in tumors of the tongue. It is likely that longer treatment time periods and higher doses of curcumin delivered through increased treatment frequency might be required to achieve beneficial effects in the other cancer patients. Cluster analysis has shown that the curcumin effect may be significant in recurrent tumors. It is also possible that some tumors are refractory to curcumin treatment because mutations in the curcumin binding site of the IKK $\beta$  protein. Because curcumin is shown to act via an AKT-independent pathway (12), cancers with AKT activation could also be immune to curcumin treatment.

We have found that IKK $\beta$  kinase activity in the salivary cells of HNSCC patients could be measured by 2 different assays described in this investigation. Because IKK $\beta$  kinase activation is an early event in the activation of NF- $\kappa$ B, enzyme activity measurements in saliva collected from patients receiving cisplatin treatment would point to the level of resistance developing through this signaling pathway in HNSCC. In such situations, a combination treatment with curcumin will be a valuable option for cancer treatment in HNSCC. We believe that IKK $\beta$  kinase activity is a good indicator of the NF- $\kappa$ B-mediated cytokine expression and thus this protein will be a useful therapeutic target in patients with head and neck cancer.

In conclusion, we believe that curcumin is a potential cancer therapeutic agent that may be combined with the current HNSCC treatment protocols to help alleviate inflammatory responses and reduce harmful side effects for patients. In the clinical setting, high doses of curcumin along with a longer treatment time period may be necessary to achieve a suppressive effect on IKK $\beta$  kinase activity, and on the expression of inflammatory cytokines. Even though high doses of oral curcumin are well tolerated in humans, there is poor absorption from the gastrointestinal tract. Hence, future studies need to be conducted to not only determine the maximum tolerated dose of curcumin, but to also find optimal delivery methods of curcumin that would more effectively treat patients with head and neck cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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