Microarray Analysis of Curcumin in HNSCC

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Abstract

Overall survival for HNSCC has not significantly improved. Treatment failure predominantly develops due to local-regional recurrences and second primaries as a result of field cancerization from tobacco use. Identifying a chemopreventive agent that alters this process could affect survival rates. Curcumin, a turmeric root extract, is in cancer clinical trials in a complex form as Curcumin C3 ®, although its effect on *in vivo* oral HNSCC has not been investigated. Understanding the cellular response to curcumin may identify a novel mechanism-based chemopreventive. Several oral HNSCC cell lines were grown in the presence of curcumin or C3 (0-40μM) and effects on growth were determined by cell proliferation assay in 96 well plates. A transcriptome profiling of a human hypopharyngeal tumor cell line exposed to curcumin for 24-48 hours was performed using Affymetrix microarrays of a human genome. Total RNA from cells was collected after 0, 24 or 48 hours of exposure to 10μM curcumin and analyzed with GeneSpring 6.1. 10μM curcumin or C3 was cytotoxic to a hypopharyngeal, poorly differentiated retromolar, and an oral cavity alveolar ridge tumor cell line, whereas 5μM was cytostatic. In the hypopharyngeal SCC, 143 genes were found to be differentially expressed after 24 hours of curcumin exposure, of which 102 genes were upregulated and 41 were downregulated. 147 genes were differentially expressed after 48 hours of curcumin exposure, of which 127 genes were upregulated and 40 genes were downregulated. HNSCC cells are sensitive to killing by curcumin. These findings provide insight into the biological effects of global gene expression and chemoprevention induced by curcumin. There has been no change in overall survival of HNSCC patients although local regional control rates have improved. ~50% of advanced stage HNSCC patients develop recurrence therefore preventing any steps of neoplastic transformation or second primaries in the UADT are a promising strategy.

Introduction

A natural product isolated from turmeric, curcumin has been implicated as a powerful therapeutic in a variety of human cancers (1) because of its ability to induce apoptosis and is currently undergoing clinical trials for colon, skin, pancreatic, and hematologic cancers, although its effect on oral HNSCC has been limited for *in vivo* benefit due to low gastrointestinal absorption (2). Laboratory data indicate that curcumin can inhibit tumor initiation, promotion, invasion, angiogenesis and metastasis. Curcumin has a long history as a food additive and is effective when applied topically to the skin or orally for colon studies but remains ineffective for systemic application. Curcumin has the desirable pharmaceutical properties including anti-inflammatory and anti-proliferation, and selectively targets rapidly dividing cells, such as those found in malignant transformation, while sparing healthy cells. Several prostrate, breast cancer and colon cancer animal models have demonstrated an ability of curcumin to inhibit tumor formation, although sufficient absorption of curcumin for clinical benefit remains problematic. Curcumin is poorly absorbed following oral administration (3) with only traces found in the blood and a majority excreted in the feces (4). Studies have shown the increased bioavailability of curcumin when administered in conjunction with the black pepper extract piperine in both rats and humans (5). This commercially available combination of curcumin and piperine, Curcumin C3 ® (Sabinsa Corp. Piscataway, NJ), is currently being evaluated in a phase III trial of metastatic colon cancer, and was selected for our studies. Curcumin has been shown to affect various cell cycle proteins and checkpoints involving downregulation of some of the cyclins and cyclin-dependent kinases, upregulation of cdk inhibitors, and inhibition of DNA synthesis (reviewed in 6) in a variety of cancers. Curcumin has previously been shown to induce a G1/S phase cell cycle arrest in the MDA 686LN HNSCC cell line (7), therefore we explored the role curcumin plays in the cell cycle from a variety of HNSCC cell lines. Curcumin is suspected to exert its effects through the mTOR pathway, a key regulator of cell growth and cell size, in prostate, glioma, rhabdomyosarcoma, and breast carcinomas (8-10). Curcumin's effects on the mTOR pathway have not been studied in HNSCC, although the modulation of NFκB, a transcription factor that controls cell proliferation and cell survival, by curcumin in HNSCC has been studied (2,11).

Methods and Materials

Cell lines and origin: FaDu-hypopharyngeal, SCC16-tongue, SCC25-tongue, SCC40-tongue, SCC066-oral cavity alveolar ridge (T4N0), SCC114-FOM (T2N0), SCC116-alveolar ridge (T2N0), PCI13-RMT (T4N1M0), PCI15a-pyriform sinus (T2N1M0), PCI30-BOT (T3N0M1). SCC cell lines kindly provided by Dr. Susan Gollin (University of Pittsburgh), and PCI cell lines kindly provided by Dr. Teresa Whiteside (University of Pittsburgh).
MTT assay: 2000 cells per well were seeded in triplicate onto 96 well plates and treated with curcumin C3 at 0, 2.5, 5, 10, 20, or 40μM for 0-72 hours. Cell viability was measured by the MTT method.
Flow cytometry: Cells were grown to ~80% confluency in the absence or presence of 10μM curcumin C3 for 0-48 hours. DNA was stained with propidium iodide after RNAse A treatment to remove RNA and analyzed by FACS Caliber (Becton Dickson).
Affymetrix: We performed a transcriptome profiling of a human hypopharyngeal tumor cell line exposed to curcumin for 24-48 hours using Affymetrix microarrays of a human genome U133A 2.0. Total RNA from cells was collected after 0, 24 or 48 hours of exposure to 10μM curcumin with subsequent microarray analysis performed with GeneSpring 6.1 software (Sillicon Genetics, Redwood City, CA). Genes that were induced or suppressed 2 fold or greater by curcumin treatment were filtered using GeneSpring software.

Results

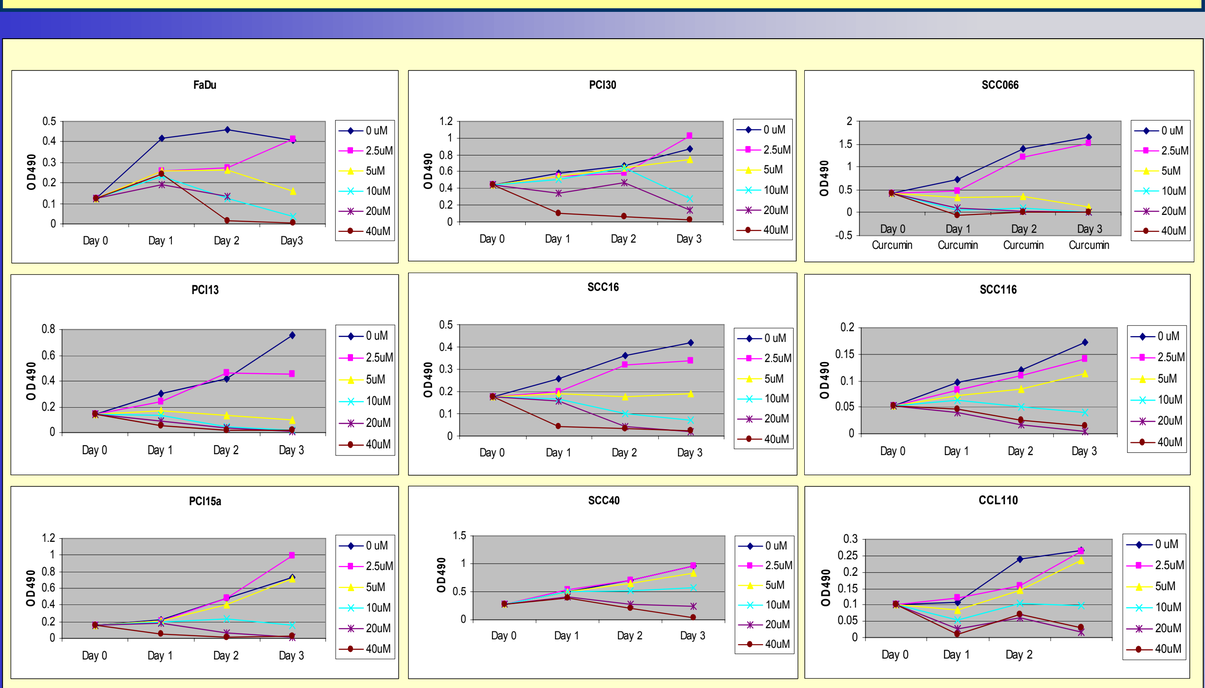

Figure 1. Growth inhibition of HNSCC cells *in vitro* with curcumin. Curcumin C3® inhibits proliferation of tumor cells FaDu, PCI-13, PCI-15a, PCI-30, SCC16, SCC40, SCC66, SCC114, SCC116, and control cells CCL110 in a dose-dependent manner. Note that cells are consistently sensitive to killing by curcumin at 5-10μM, a physiologically relevant concentration.

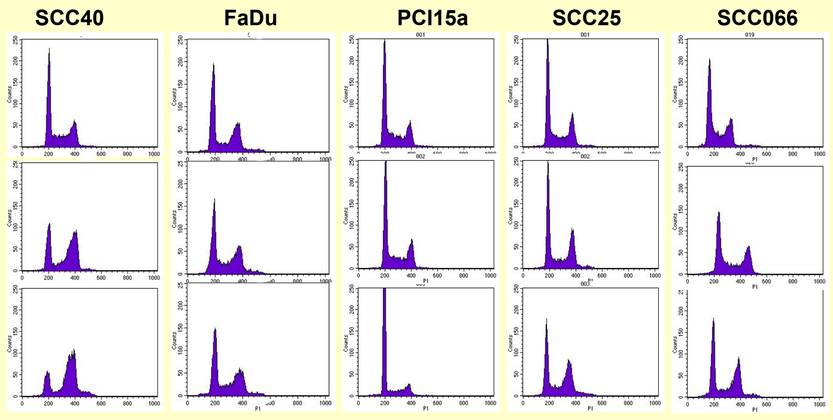

Figure 2. Flourescence activated sorting (FACS) analysis of HNSCC cells treated with curcumin. FACS analysis was done on HNSCC cell lines treated with 10μM C3, a concentration inducing cell death in all cell lines analyzed by MTT assay. A peak at 200 corresponds to DNA in the G1 phase of the cell cycle, and a peak at 400 corresponds to the G2 phase of the cell cycle. Note that the SCC40 histogram depicts a G2/M arrest whereas the PCI15a histogram corresponds to a G1/S cell cycle arrest, while other cell lines do not convincingly demonstrate any cell cycle arrest with curcumin treatment.

Results

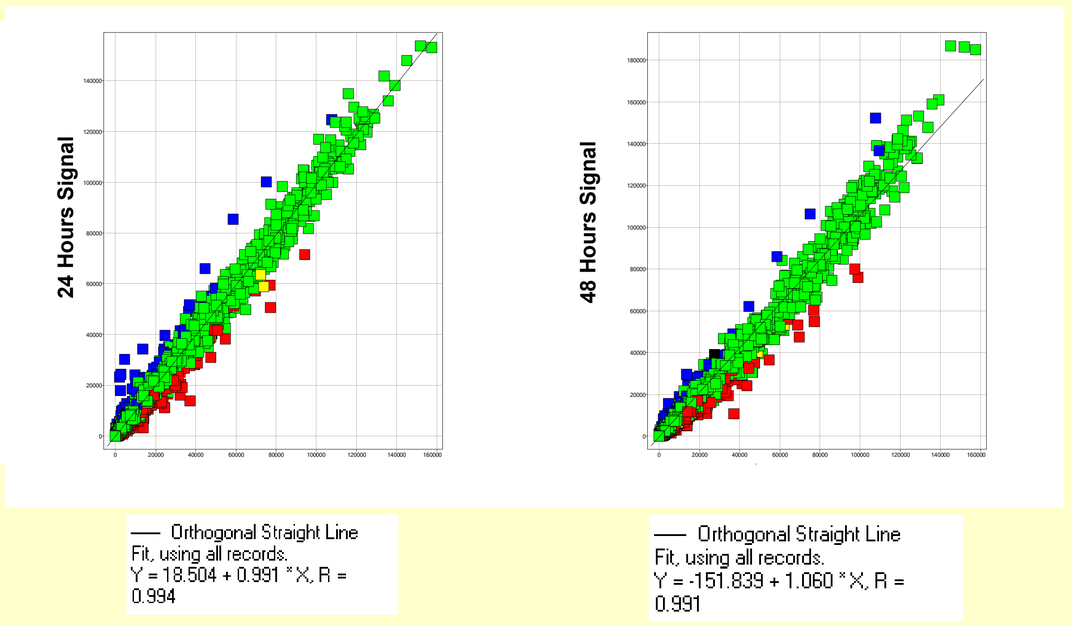

Figure 3. Scatter plot analysis of differentially expressed genes in response to curcumin treatment. Over-expressed genes are indicated by blue, under-expressed by red, moderate increase by black, moderate decrease by yellow, and no change by green. Several signaling molecules, transcription factors, and mTOR pathway components were identified that changed expression upon curcumin treatment.

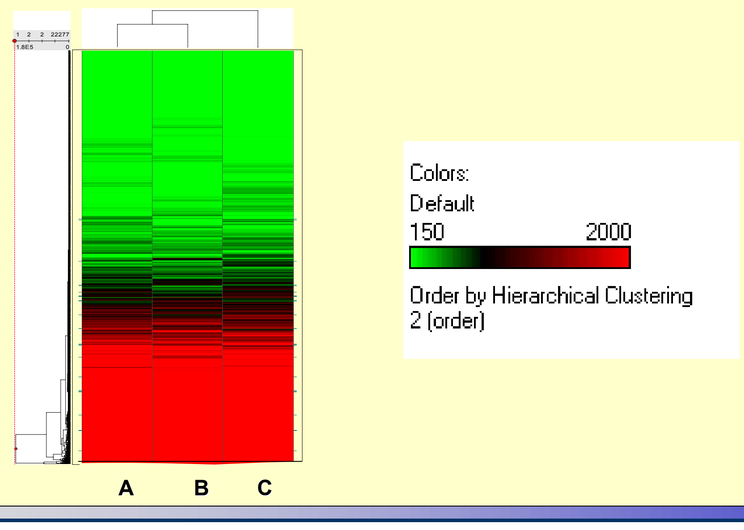

Figure 4. Hierarchical clustering of curcumin treatment. 22277 (All records) were analyzed and clustered by UPGMA (unweighted average) with a similarity measure of Euclidean distance and ordered by average value. **A.** 24 hours curcumin treatment. **B.** Control cells. **C.** 48 hours curcumin treatment. Note a difference in clustering patterns of genes after 24 and 48 hours curcumin treatment indicating a change in gene expression.

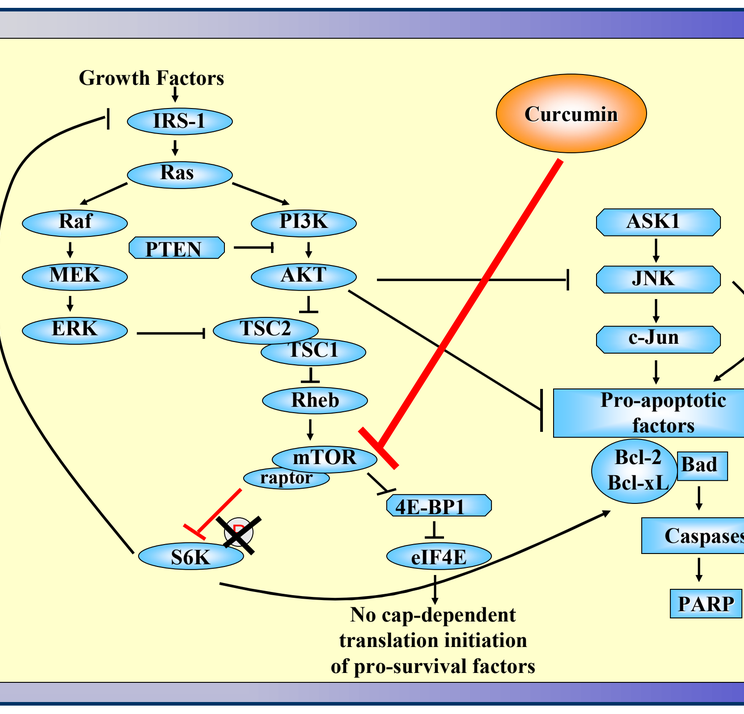

Figure 5. mTOR signaling pathway. Curcumin inhibits the proliferation of tumor cells by inhibiting the mTOR pathway (8). The mTOR pathway is a key signaling pathway that regulates cap-dependent translation as well as apoptosis through phosphorylation of S6K and subsequent de-phosphorylation of Bad, which allows association of Bcl-2/Bcl-xL to initiate activation of the caspase pathway and cleavage of PARP.

Conclusions

A total of 143 genes were found to be differentially expressed after 24 hours of curcumin exposure, of which 102 genes were upregulated and 41 were downregulated. A total of 147 genes were found to be differentially expressed after 48 hours of curcumin exposure, of which 127 genes were upregulated and 40 genes were downregulated. Some of the most notable differentially expressed genes were involved in the cell cycle (Fig 2), NFκB (and therefore apoptosis/cell survival), phosphorylation of 4EBP1, oxidative state of the cell, G protein coupled receptors (GPCR), cell morphology/motility, extracellular matrix, and vesicle trafficking. These results indicate that curcumin affects many components of the mTOR and mTOR-related signaling pathways but does not affect mTOR directly at the level of transcription (8, and data not shown). The mammalian target of rapamycin (mTOR) is at the center of a signaling pathway that mediates proliferation and cell survival, and has become a target for anticancer therapies through the use of rapamycin and its derivatives (12). Targeting the mTOR pathway with rapamycin and rapamycin analogues such as CCI-779, RAD 001, and AP23573 inhibits cell cycle progression, cell growth, and proliferation signaling. In cancer cells, these pathways may be constitutively activated. Clinical trials have demonstrated tumor growth suppression against a variety of human cancers with rapamycin and its analogues. Therefore, development of modulators of mTOR and related pathways is a rational chemopreventative strategy for malignancies with a wide range of molecular abnormalities.

Bibliography

1. Aggarwal, BB et al *Anticancer Research* 2003; **23:**363-98.
2. LoTiempo et al *Clinical Cancer Research* 2005; **11:**6994-7002.
3. Ammon, HPT et al *Planta Medica* 1999;**57:**1-7.
4. Ravindranath, V 1980 *Toxicology* **20:**251-257.
5. Shoba G. *Planta Medica* 1998, **64:**353-356.
6. Karunagara D. *Advances Experimental Medical Biology* 2007; **595:**245-68
7. Aggarwal S. *International Journal of Cancer* 2004, **111(5):**679-92.
8. Beevers CS, *International Journal of Cancer* 2006, **119(4):**757-64.
9. Li M, *Cancer Research* 2007, **67(5):**1988-96.
10. Aoki H, *Molecular Pharmacology* 2007, **72(1):**29-39.
11. Aggarwal S., *International Journal of Cancer* 2004, **111(5):** 679-92.
12. Vignot S. *Annals of Oncology* 2005, **16:**525-537.