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Curcumin reactivates epigenetically silenced tumor suppressor gene tissue factor pathway inhibitor-2 in hepatocellular carcinoma cells.

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CURCUMIN REACTIVATES EPIGENETICALLY SILENCED TUMOR
SUPPRESSOR GENE TISSUE FACTOR PATHWAY INHIBITOR-2 IN
HEPATOCELLULAR CARCINOMA CELLS

By

Akshata Moghe
M.B.,B.S., Mumbai, India, 2007

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ABSTRACT

CURCUMIN REACTIVATES EPIGENETICALLY SILENCED TUMOR
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Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most fatal, with a rising incidence in the US as a result of the increase in alcoholic liver disease and obesity. Current therapeutic strategies are unsatisfactory with poor treatment outcomes and efficacious therapies are acutely needed for better management of this deadly disease. Curcumin, a phenolic compound from the rhizome of the plant Curcuma longa has been shown to inhibit growth and induce cell death in various types of cancer cells including HCC. However, the anti-HCC mode of action of curcumin has not yet been elucidated.

In HCC, aberrant promoter methylation and histone deacetylation are implicated in the inactivation of tumor suppressor genes which has a significant impact on carcinogenesis. Tissue factor pathway inhibitor-2 (*TFPI-2*), a Kunitz-type serine protease inhibitor, is a tumor suppressor gene that is frequently epigenetically silenced in human HCC and HCC cell lines. Restoration of TFPI-2 expression in tumor tissue has been shown to not only inhibit invasion, tumor

growth, metastasis and angiogenesis but also induce apoptosis. We examined the effects of curcumin on tumor suppressor genes in HCC and observed robust reactivation of TFPI-2 in HepG2 cells upon curcumin treatment. We confirmed that TFPI-2 was under epigenetic control in HepG2 cells by using demethylating agent 5-Aza-2'deoxycytidine (5-AZA) and histone deacetylase inhibitor trichostatin A (TSA), both of which induced gene expression. Further, we investigated the epigenetic modifications at the TFPI-2 promoter and the alterations induced by curcumin in these epigenetic states. Histone H3 acetylation at the transcription factor binding sites of the TFPI-2 promoter region was increased by curcumin and correlated with the induction of gene expression. The reactivation of TFPI-2 also corresponded with a decrease in cell invasiveness and increase in cell death in HepG2 cells. Overall, our data strongly suggest that curcumin can reverse the epigenetic alterations at the TFPI-2 promoter and thus, reactivate this silenced tumor suppressor gene. These results support a potential therapeutic role for curcumin in the management of HCC.

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CHAPTER I

INTRODUCTION

Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world[1-2] and the third most common cause of cancer-related mortality[1, 3]. Every year, between 500,000 and 1000,000 new cases of HCC are detected, and almost all of the victims die of the disease. HCC has a higher incidence in developing countries, especially in Sub-Saharan Africa and South Asia, due to the increased prevalence of viral hepatitis in these regions. However, this trend is now changing and there has been an alarming rise in the incidence of HCC in economically developed nations like Japan, Western Europe and the United States in the past few years[2]. In the United States, in addition to Hepatitis C viral (HCV) infection, the growing epidemics of alcoholic liver disease (ALD), obesity and non-alcoholic steatohepatitis (NASH) have contributed to the rapid rise in HCC incidence. Liver cirrhosis is a premalignant condition that greatly increases the risk of HCC, and is common in the USA[2]. It is expected that the incidence of HCC will only continue to rise in the coming years as latent HCV infections come to the fore and as more and more cases of ALD and NASH

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emerge and progress to cirrhosis[2-3]. The reason for the high mortality associated with HCC is that the treatment modalities available today are minimally effective at best. The best treatment option is liver transplantation, which is not only expensive but also complicated in the setting of liver cirrhosis, a condition invariably present in these patients. Moreover, most patients are diagnosed at a late stage in the disease, and their cancers are not amenable to transplantation. Other surgical interventions such as liver resection, percutaneous ethanol injection, radiofrequency ablation, transarterial embolization and chemoembolization have been tried with unsatisfactory outcomes. Adjuvant chemotherapy with various chemotherapeutic agents such as doxorubicin, cisplatin, fluorouracil and interferon also yields little survival benefit. Recently, Sorafenib, a tyrosine protein kinase inhibitor, was approved for use in HCC but its efficacy and toxicity is still being investigated in clinical trials. If left untreated, HCC progresses rapidly to death within 6-8 months even when diagnosed early. Patient prognosis is poor and the 5-year survival rate utterly dismal at less than 5 percent[4]. As a result, there is an urgent need for the development or discovery of a novel, effective therapy for the management of HCC.

Pathogenesis of HCC : relevance to tumor suppressor genes

An understanding of the process of hepatocellular carcinogenesis is critical in order to define pertinent molecular targets for therapy. Until now, HCC had been linked to increases in allelic losses, chromosomal changes, gene mutations and alterations in molecular cellular pathways[5]. It is now recognized that in addition to the known genetic alterations of mutations and deletions, epigenetic alterations can also play a major role in tumorogenesis[5]. In HCC, as in other cancers, aberrant DNA methylation and specific histone modifications are implicated in the development and progression of the disease[5-7]. Aberrant DNA methylation occurs in the form of global hypomethylation and regional hypermethylation of CpG islands. The global hypomethylation results in the activation of protooncogenes. Regional hypermethylation in the promoter regions of tumor suppressor (TS) genes leads to their transcriptional silencing and inactivation[6, 8]. Several TS genes such as TFPI-2, RASSF1A, P16, P15 and GADD45 have been found to be frequently epigenetically silenced through promoter hypermethylation in HCC[8-13].

Fig.1 Epigenetic mechanisms of carcinogenesis in HCC

Tissue Factor Pathway Inhibitor-2 (TFPI-2)

Amongst the identified TS genes, **Tissue factor pathway inhibitor-2 (TFPI-2) is significantly underexpressed**[14] **in approximately 90 percent of primary HCCs when compared to their corresponding non-tumorous livers**[9]. Wong et. al. (2007) studied the gene expression profiles of several candidate genes in HCC by microarray analysis and detected frequent inactivation of TFPI-2 in several human HCC cell lines and human HCC samples. Expression of TFPI-2 in HCC cell lines could be robustly restored by treatment with known epigenetic modulators such as 5-Aza-2-deoxycytidine (5-AzadC, a DNA methyltransferase inhibitor) and Trichostatin A (TSA, a histone deacetylase inhibitor) , which implied that the gene was indeed under epigenetic control[9].

TFPI-2 is a Kunitz-type serine proteinase inhibitor. Previously designated as placental protein 5, it inhibits a broad range of serine proteinases and plays an important role in the regulation of extracellular matrix degradation and remodeling[15]. TFPI-2 inhibits plasmin and trypsin activity and reduces cellular invasiveness of tumors. Reduced expression of TFPI-2 has been associated with several patho-physiological processes such as inflammation, angiogenesis, atherosclerosis, retinal degeneration and tumor growth and metastasis[15]. Besides HCC, TFPI-2 is inactivated in many other tumors, including esophageal, pancreatic, gastric, colon and breast cancers[16-20]. Overexpression of TFPI-2 or upregulation of the reduced expression of TFPI-2 has the potential to limit tumor growth by inhibiting angiogenesis, cellular proliferation and cellular invasiveness of tumor cells[21-22]. Restoration of TFPI-2 has also been shown to trigger

apoptosis through a caspase-mediated mechanism in a human glioblastoma cell line[23].

ABERRANT CpG ISLAND METHYLATION
and/or
UNFAVORABLE HISTONE MODIFICATIONS
at theTFPI-2 promoter

TFPI-2

Plasmin

Other serine proteases

Matrix Degradation

Pro-MMPs

MMPs

MMPs: Matrix metalloproteinases

- Cell migration/cell invasiveness
- Metastasis
- Angiogenesis
- Vasculogenesis
- Inflammation
- Atherosclerosis
- Release of growth factors and cytokines

Fig.2 Proposed epigenetic mechanisms that silence tumor suppressor gene TFPI-2 in HCC and the implications of inactivation of the gene Modified from Chand HS et al.[15]

Importantly, in HCC as well, ectopic overexpression of TFPI-2 has been shown to significantly suppress proliferation and invasiveness of tumor cells[9]. Taken together, these data strongly imply that loss of TFPI-2 expression is critical to the establishment and progression of HCC. **Hence, TFPI-2 is an important target tumor suppressor gene in the treatment and control of HCC.**

Curcumin as an epigenetic modulator

Curcumin, the phenolic compound from the rhizome of the plant *Curcuma Longa* (turmeric, yellow curry powder) has been used for centuries in ancient medicine to treat various diseases. In recent years, curcumin (diferuloylmethane) has been extensively characterized to define the mechanisms underlying its innumerable beneficial properties. Curcumin displays a plethora of favorable effects, including anti-inflammatory, anti-oxidant, anti-proliferative and pro-apoptotic effects[24-25]. Its anti-inflammatory effects are mediated through the inhibition of inflammatory transcription factors (such as nuclear factor κB), enzymes (such as cyclooxygenase-2 and 5-lipoxygenase) and cytokines (such as tumor necrosis factor, interleukin 1 and interleukin 6)[25]. Since most chronic diseases involve inflammation, curcumin has been used to treat a myriad of cardiovascular, pulmonary, neurological and metabolic diseases. The anti-tumorigenic properties of curcumin are being investigated in many cancers[26] and curcumin is also being tested in human clinical trials for the treatment of colon

and pancreatic cancers[27]. Importantly, curcumin has been shown to possess anti-HCC properties in *in vitro* and in animal studies[28-29]. However, its exact mechanism of action in the control of HCC is still not well-elucidated.

In our experiments, we confirmed that curcumin kills HCC cells using HepG2, a well characterized HCC cell line. We also examined the effect of curcumin on TFPI-2 in HepG2 cells and our preliminary data show that curcumin was able to significantly reactivate TFPI-2 gene expression, similar to the effects of the epigenetic modulators 5-Aza-2-deoxycytidine and Trichostatin A. These results support a potential role for curcumin as an epigenetic modulator in its anti-HCC action. Curcumin has recently been shown to cause epigenetic alterations through both, histone modifications and DNA methylation[30]. However, these alterations induced by curcumin are only beginning to be studied.

Hypothesis

The effects of curcumin appear to be complex and context-dependent as curcumin supports histone acetylation in some cases and histone deacetylation in others[31-32]. Moreover, there is no systematic study as yet investigating the role of curcumin as an epigenetic modulator in HCC cells. Based on our preliminary data, we propose that curcumin induces epigenetic modifications in HCC cells, particularly with respect to tumor suppressor genes. Specifically, we hypothesize that **curcumin induces de-repression of the epigenetically silenced tumor**

suppressor gene TFPI-2 in hepatocellular carcinoma (HCC) cells and that

this effect is a significant component of its anti-HCC action.

CHAPTER II

MATERIALS AND METHODS

Reagents

Curcumin was purchased from Biomol (Plymouth Meeting, PA). 5-Aza -2' deoxycytidine, Trichostatin A and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). DMEM, penicillin, streptomycin and TRIzol® were obtained from Invitrogen (Carlsbad, CA). All RT-PCR reagents were purchased from Applied Biosystems (Foster City, CA). MTT (Thiazolyl Blue Tetrazolium Bromide) was obtained from Sigma-Aldrich (St. Louis, MO) and the CellTiter-Glo® Luminescent Cell Viability assay was obtained from Promega Corporation (Madison, WI). The CytoSelect™ 24-Well Cell Invasion Assay was purchased from Cell Biolabs, Inc. (San Diego, CA).

Cell Culture

HepG2 cells (clone E6-1, ATCC, Rockville, MD) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 10 µg/ml streptomycin. Cells were maintained in an incubator at 37°C with humidified 5% CO_2 . Cells were plated and incubated overnight before treatment, in all experiments.

RNA Isolation and Real-Time PCR Analysis

Total RNA was isolated from cells treated with curcumin, 5-AZA or TSA using TRIzol®, according to manufacturer's instructions. For real time PCR, the first strand cDNA was synthesized using TaqMan Reverse transcription reagents (Applied Biosystems). The reverse transcription was carried out using 1X Taqman RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM random hexamer, 8 U of RNase inhibitor and 25 U of Multiscribe Reverse Transcriptase with 200ng of total RNA. The RT conditions were 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. Reactions in which the RNA was omitted served as negative controls. Real time PCR was performed with an ABI prism 7500 sequence detection system and SYBR green I dye reagents.

Reverse transcriptase polymerase chain reaction (RT-PCR) assays were used to assess TFPI-2 mRNA levels in HepG2 cells. TFPI-2 and 18S rRNA specific primers were purchased from SuperArray Bioscience Corporation (Frederick, MD). The parameter threshold cycle (C_t) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method by normalizing with 18S rRNA gene expression in all the experiments.

Cell Survival Assays

The MTT assay and the CellTiter-Glo® Luminescent Cell Viability assay were used to assess cell survival. For the MTT assay, cells were plated in 96 well microplates, incubated overnight and treated with curcumin for 24 hours.

Treatments were then removed and MTT (1mg/ml) was added in fresh DMEM (100 µl/well). After incubation at 37°C with humidified 5% CO_2 for 1-3 hours, MTT lysis buffer (100 µl/well) was added. The cells were further incubated overnight for complete lysis. Cell viability was quantified colorimetrically by measuring O.D. at 570 nm. The CellTiter-Glo® Luminescent Cell Viability assay was used to determine the number of viable cells based on the amount of ATP present. Briefly, 10,000 cells/100µl per well were plated in 96 well plates. After treatment with different concentrations of curcumin (6.25 to 50 µM) for 24 hours, an equal volume (100µl) of CellTiter-Glo® reagent was added to each well. Plates were incubated at room temperature for 10min to allow complete cell lysis and stabilization of luminescence. The Luminescent signal (RLU) was measured by Orion luminometer.

Cell Invasion Assay

The CytoSelect™ 24-Well Cell Invasion Assay was performed as per manufacturer's instructions. Briefly, a suspension of 1x 10^6 cells/ml was prepared in serum free media and 300 µl of this suspension was placed in the upper chamber on the rehydrated membrane. 500 µl of culture medium with 10%fetal bovine serum (chemoattractant) was placed in the lower chamber. The chamber-insert assembly was allowed to incubate for at 37°C in 5% CO_2 atmosphere. After 24 hours, the non-invasive cells were removed, the inserts stained, washed and allowed to air dry. Each insert was then incubated in Extraction Solution on

an orbital shaker at room temperature. O.D. was measured using a colorimetric plate reader.

Chromatin Immunoprecipitation (ChIP) and qChIP PCR analysis

The ChIP assay was conducted using the ChIP assay kit according to the manufacturer's instructions (Millipore, Billerica, MA). Briefly, 10×10^6 cells were crosslinked with 1% formaldehyde- 1 X-glycine and lysed in lysis buffer containing protease inhibitor cocktail. Enzymatic digestion of DNA was performed as per manufacturer's protocol (EZ-Zyme Magna CHIP KIT) to produce chromatin fragments of 100–166 bp. ChIP antibody directed against acetyl-histone H3 (Lys9, 14) (Millipore, Billerica, MA), was used for immunoprecipitation. The lysates were incubated overnight with antibody, following which the antibody/protein complexes were immunoprecipated using magnetic beads. Successive washes were performed with low salt, high salt, LiCl and Tris EDTA (TE) wash buffers. After reverse crosslinking and purification, pure DNA was eluted in elution buffer (1% SDS, 0.1 M NaHCO3). qChIP-PCR was performed using the ABI Prism 7500 Sequence Detection System. ChIP-PCR primers designed for two regions of the TFPI-2 promoter were used and their sequences are as follows:

ChIP TFPI-2 (I) LP   GGCTTCTTTACAGCGCAATC

            RP   ACCTGCCTCCCAAACTTTCT

ChIP TFPI-2 (II) LP   ACAGTCCCCGTGCATGAAT

            RP   GCAAGGCGTCCGAGAAAG

Data was analyzed as Differential Occupancy Fold Change. ChIP-qPCR results were calculated by ΔΔCt method (since all Ct values for a binding site are determined from the same qPCR assay) where each ChIP DNA fractions Ct value was normalized to the Input DNA fraction Ct value using ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log2 (Input Dilution Factor))). The difference between the normalized experimental sample (S2) and the control sample (S1) ChIP fraction Ct values (second ΔΔCtt) was determined using ΔΔCt [S2-S1] = ΔCt [S2: normalized ChIP] - ΔCt [S1: normalized ChIP].

Bisulfite treatment and methyl-specific PCR

DNA was extracted from untreated and curcumin-treated cells using the DNeasy tissue kit and DNA samples were treated with bisulfite (EZ DNA methylation Gold Kit from Zymo) following the manufacturer's protocol. Human unmethylated DNA (Qiagen) and human methylated DNA (Active Motif) were used as controls. The bisulfite-converted samples and controls were then amplified with primer pairs specific for bisulfite converted methylated DNA and unmethylated DNA .The PCR reaction uses 10 ng of the bisulfite treated DNA in a 25 µl reaction. Standard PCR conditions were employed at an optimized annealing temperature. The products were analyzed on a 1.4% agarose gel. The methylation-specific primer sets have been derived from experiments performed by Glockner et. al.[19] and have been tested in our laboratory. The primer set most sensitive to methylation differences at positions -93 and -32 (with respect to the TSS) of the TFPI-2 promoter was used, and the primer sequences for this set are shown below. 'M'

stands for the methylated DNA-specific primer and 'U' stands for the unmethylated DNA-specific primer:

TFPI2(Glockner)-M(1)-L	CGGCGGGGTGATAGTTTTC
TFPI2(Glockner)-M(1)-R	CAAACGACCCGAATACCCGCTTTATACG
TFPI2(Glockner)-U(1)-L	GTGGTGGGGTGATAGTTTTTG
TFPI2(Glockner)-U(1)-R	CCAAACAACCCAAATACCCACTTTATACA

Table (1) Methyl-specific Real-Time PCR primers for a single region of the TFPI-2 promoter

Statistical Analysis

Data are presented as means± SD for the indicated number of independently performed experiments. Student's t-test and one-way ANOVA with Tukey's multiple comparison test were used for the determination of statistical significance. $P<0.05$ was considered significant.

CHAPTER III

RESULTS

Curcumin reactivates TFPI-2 in HepG2 cells

TFPI-2 is an important tumor suppressor gene that is significantly underexpressed in most HCC cell lines and primary HCCs. Hence, this gene likely plays a key role in the development and establishment of HCC. We therefore examined whether treatment with curcumin affects the expression of this critical tumor suppressor in HepG2 cells

HepG2 cells were plated at a density of 0.5×10^6 cells/ml and incubated overnight under standard conditions. The cells were then treated with 25 µM curcumin for 24 hours and RNA was obtained as described earlier, for RT-PCR analysis.

Curcumin induced a robust expression of TFPI-2 at 24 hours (fig. 3).
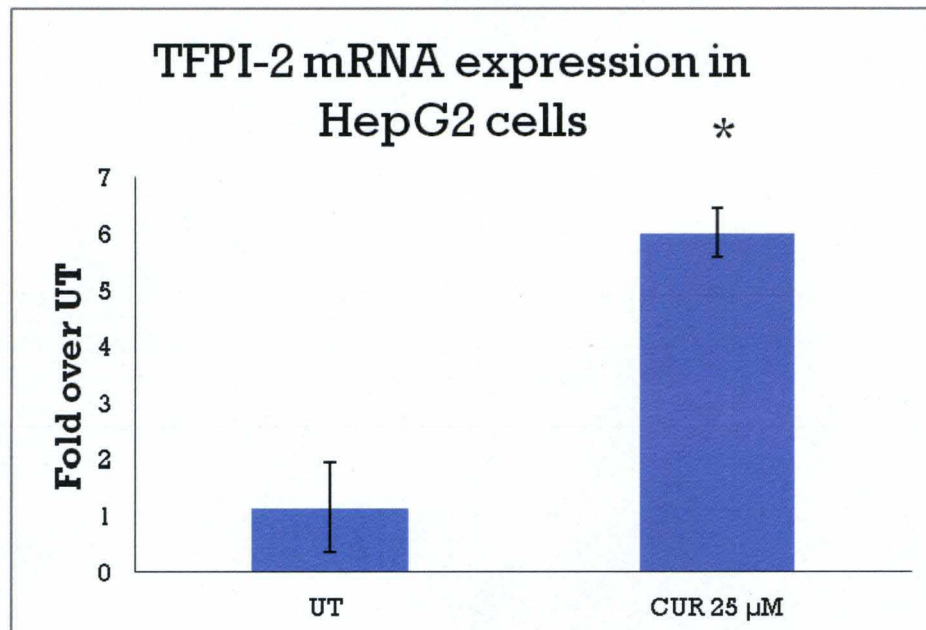
Fig. 3 Curcumin-induced TFPI-2 expression in HCC cells.

HepG2 cells were left untreated (UT) or treated with curcumin (25 µM) for 24 hours. TFPI-2 mRNA expression is normalized to 18S rRNA expression and shown as fold over untreated. Data is presented as mean ± SD, n=3; * $p<0.05$ when compared to UT

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TFPI-2 is under epigenetic control in HepG2 cells

It has been shown in previous studies that TFPI-2 is frequently repressed in HCC cells by epigenetic mechanisms such as DNA methylation and histone deacetylation. We examined the epigenetic regulation of TFPI-2 in HepG2 cells using two known epigenetic modulators, 5-Aza-2'deoxycytidine (5-AZA) and Trichostatin A (TSA). 5-AZA is a DNA methyltransferase (DNMT) inhibitor and functions as a demethylating agent. TSA is a histone deacetylase (HDAC) inhibitor and allows acetylation of histone residues. 5-AZA and TSA thus, promote transcriptionally permissive epigenetic states, by causing CpG island demethylation and histone acetylation, respectively, at promoters of epigenetically regulated genes.

Both 5-AZA and TSA derepressed TFPI-2 leading to the induction of gene expression, demonstrating that TFPI-2 is indeed controlled by histone acetylation and DNA methylation in HepG2 cells (fig. 4). In comparison to 5-AZA, TSA induced TFPI-2 expression to a greater extent, suggesting that histone acetylation likely plays a predominant role in regulating the transcriptional induction of TFPI-2 gene. Further experiments at longer time points are required to completely characterize the effects of these agents.

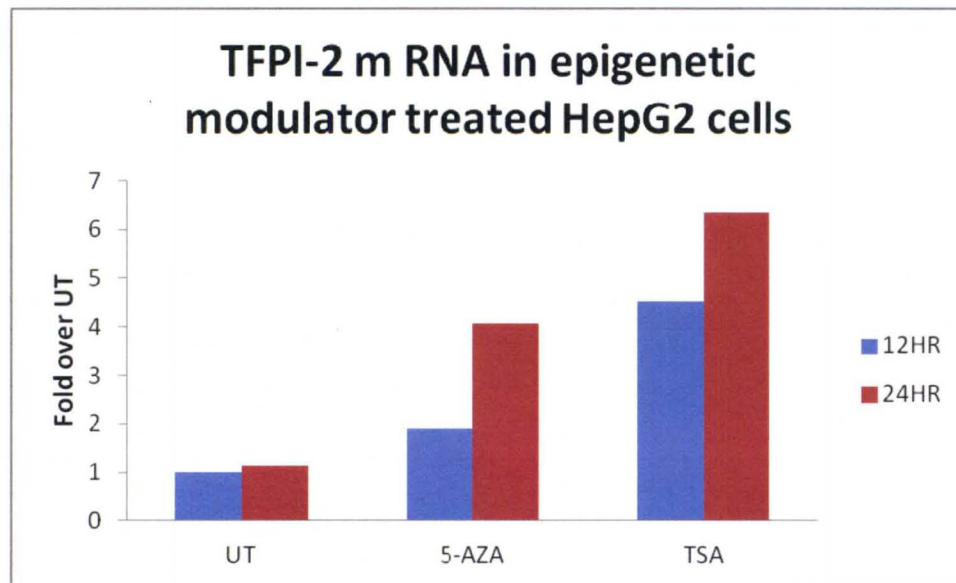**TFPI-2 m RNA in epigenetic modulator treated HepG2 cells**

Fig. 4 TFPI-2 is epigenetically regulated in HepG2 cells

HepG2 cells were left untreated (UT) or exposed to 5-Aza-2'deoxycytidine 10 μM (5-AZA) or Trichostatin A 300 ng/ml (TSA) and TFPI-2 mRNA expression was examined at 12 and 24 hours by RT-PCR analysis. TFPI-2 mRNA expression was normalized to 18S expression and shown as fold over untreated.

Curcumin increases histone H3 acetylation at the TFPI-2 promoter

Since TFPI-2 is found to be epigenetically silenced in HepG2 cells, we hypothesized that curcumin utilizes epigenetic mechanisms to derepress the gene. Curcumin likely modifies or reverses the transcriptionally repressive epigenetic state of the TFPI-2 promoter. To test this hypothesis, we first examined the acetylation status of the histone H3 at the TFPI-2 promoter using the Chromatin Immunoprecipitation (ChIP) assay. The ChIP technique allows for investigation of DNA–protein interactions at the regions of interest in a gene promoter. We designed primers that would specifically amplify TFPI-2 gene promoter regions that have binding sites for transcription factors such as AP-1, SP-1 and Egr-1, which are relevant for TFPI-2 gene expression. As shown in fig. 5, Region I is closer to the transcription start site (TSS) and covers binding sites for AP-1, SP-1 and Egr-1. Region II is further away from the TSS and covers an area with several binding sites for AP-1.
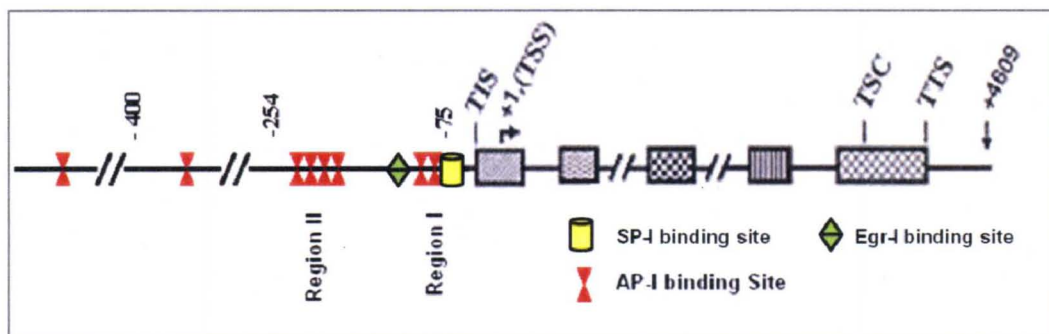

Fig.5 Schematic illustration of the TFPI-2 gene and the regions amplified by the ChIP primers (Regions I and II)

TIS, transcription initiation site; TSS, translation start site; TSC, translation stop codon; TTS, transcription termination site. Modified from Chand HS et al.[15]

In this study, we investigated whether curcumin induces changes in the histone H3 acetylation at the TFPI-2 promoter, that are relevant for the regulation of transcription. Specifically, histone H3 acetylation at lysines 9 and 14 (H3Ac) was examined. This modification is transcriptionally permissive in the promoter region and represents 'open chromatin'. Cells were plated and treated with curcumin as described in earlier experiments. At 24 hours, the cells were fixed with formaldehyde, enzyme digested, and immunoprecipitated with anti-H3Ac antibody. DNA obtained after immunoprecipitation was reverse cross-linked and amplified with the RT-PCR primers mentioned above (methods). TSA, being an HDAC inhibitor, was used as a control in these experiments.

Fig.6 Curcumin increases histone H3 acetylation at the TFPI-2 promoter (Region I)

Cells were left untreated (UT) or treated with curcumin (25 µM) or TSA (300 ng/ml) for 24 hours. Chromatin was collected and ChIP RT-PCR was performed

for detection of H3Ac at Region I of the TFPI-2 promoter. The data is a representation of three independent experiments.
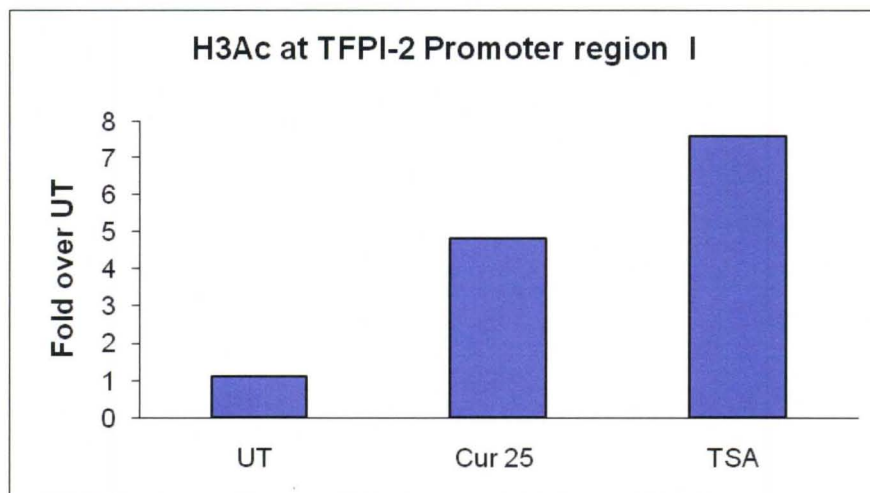

Fig.7 Curcumin increases histone H3 acetylation at the TFPI-2 promoter (Region II)

Cells were left untreated (UT) or treated with curcumin (25 μM) or TSA (300 ng/ml) for 24 hours. Chromatin was collected and ChIP RT-PCR was performed for detection of H3Ac at Region II of the TFPI-2 promoter. The data is a representation of three independent experiments.
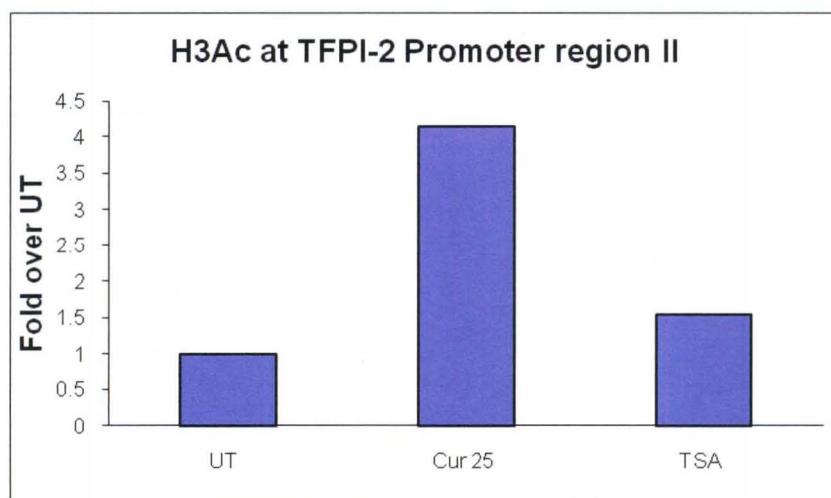
CpG island methylation at nucleotide positions -93 and -32 of the TFPI-2 promoter is unaffected by curcumin

Another epigenetic mechanism that contributes to silencing of genes is hypermethylation of the cytosines of the CpG sites in gene promoter regions. TFPI-2 promoter methylation has been detected in 80% of HCC cell lines and 47% of human HCCs in a study by Wong et. al.[9]. Aberrant promoter methylation has also been shown to correlate with silencing of TFPI-2 in gastric and pancreatic tumors. Hence, it is important to examine the effect of curcumin on the methylation CpG sites in the TFPI-2 promoter.

Bisulfite conversion of DNA and methyl-specific RT-PCR was used to examine the methylation status of CpG sites at nucleotide positions -93 and -32 of the TFPI-2 promoter, as described earlier in the methods chapter. Completely methylated human DNA and completely unmethylated human DNA were used as controls. The TFPI-2 promoter was found to be mostly unmethylated at the sites investigated by the primer set used as indicated by the faint band of methylated DNA. Further, there was no observable difference in the faint methylated DNA band after treatment with (25 µM) curcumin for 24 hours (fig. 8)

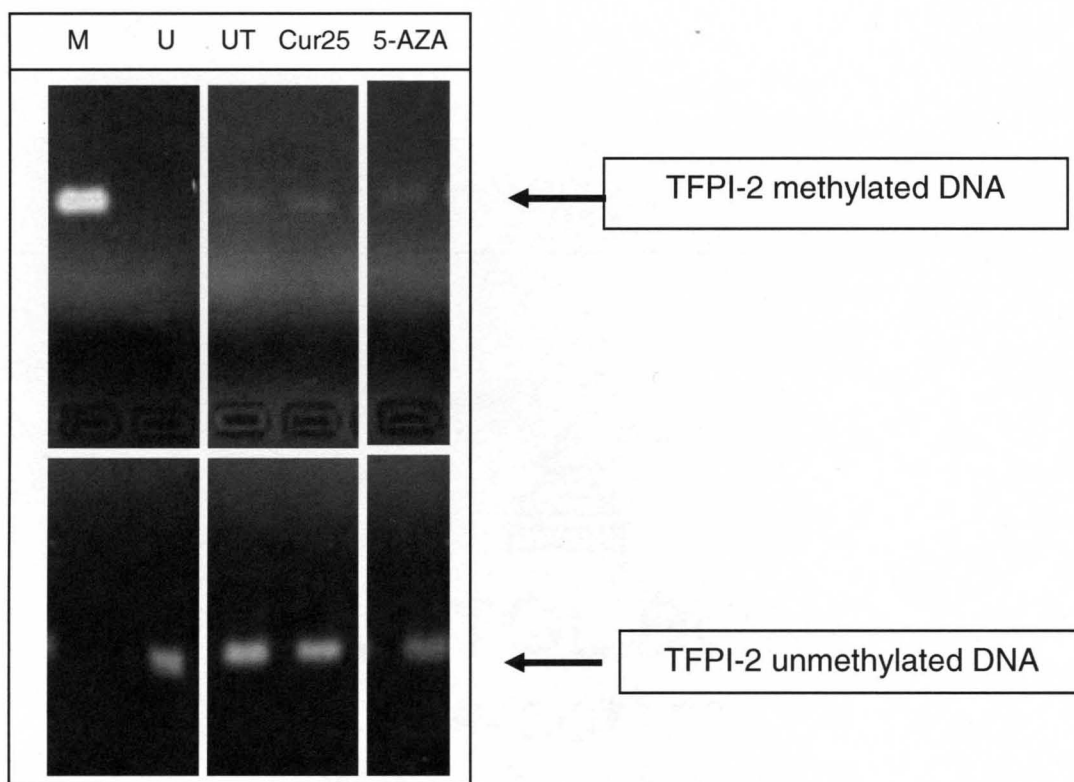**Fig. 8 TFPI-2 is mostly unmethylated at nucleotide positions -93 and -32 in the promoter region**

Cells were left untreated (UT) or treated with curcumin (25 µM) for 24 hours. DNA was collected, bisulfite converted and amplified by methyl-specific PCR. Methyl-specific primer sets for a single site in the TFPI-2 promoter were used. M = Human methylated DNA control, U = Human unmethylated DNA control

Curcumin reduces cell invasiveness of HepG2 cells

TFPI-2 is a matrix-associated Kunitz-type serine proteinase inhibitor, and plays an important role in normal extracellular matrix (ECM) remodeling. The proteinases inhibited by TFPI-2 in the ECM are not known with certainty. However, studies have shown that TFPI-2 inhibits plasmin *in vitro* and most likely *in vivo*. Recent reports have also demonstrated that TFPI-2 probably inhibits matrix metalloproteinases directly in the ECM. Loss of TFPI-2 thus allows for degradation of the ECM, which is a critical step in the process of tumor invasion and spread.

In order to study the effect of curcumin on cellular invasiveness in HCC, we performed a cell invasion assay. HepG2 cells were plated, treated with 15 µM curcumin for 24 hours and allowed to pass through a rehydrated basement membrane as per manufacturer's protocol (see methods). Curcumin reduced cell invasion across the membrane, as shown by the stained inserts and by colorimetric quantification in fig. 9.

9 (a)

HepG2 Cell Invasion at 24 hours

Untreated   Curcumin 15 uM

9 (b)

HepG2 Cell Invasion Assay

O.D.

UT   Cur 15 uM
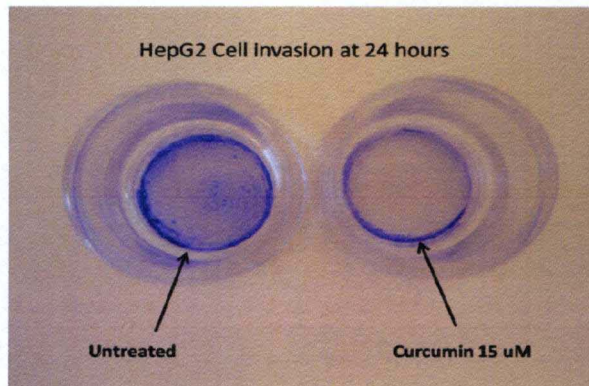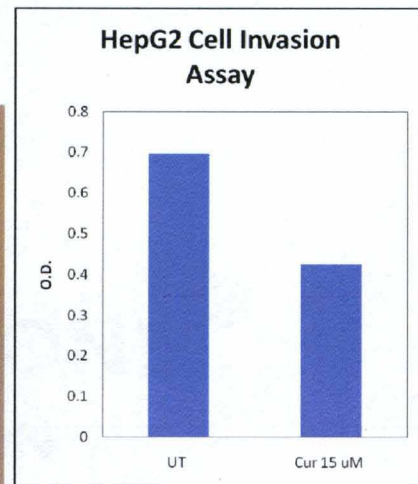
Figs. 9 (a) and (b) Curcumin reduces cell invasiveness in HepG2 cells

Cells were left untreated (UT) or treated with 15 µM curcumin and allowed to invade a rehydrated basement membrane in the cell invasion assay. Staining of invasive cells is shown in fig. 9 (a). The invaded cells were then collected, lysed and a colorimetric quantification was performed, as shown in fig. 9 (b).

Curcumin reduces HepG2 cell survival

Restoration of TFPI-2 in tumors inhibits cell proliferation and angiogenesis, besides reducing cellular invasiveness[9]. Ectopic overexpression of TFPI-2 has also been shown to reduce cell growth in HCC cell lines[9]. TFPI-2 triggered apoptosis in a glioblastoma cell line, probably via a caspase-mediated mechanism[23].

We examined the effects of curcumin on cell survival in HepG2 cells using two assays – the MTT assay and the CellTiter-Glo® Luminescent Cell Viability assay. The MTT assay measures the activity of enzymes that reduce MTT to purple formazan, and thus, is a measure of viable and active cells. Compared with untreated cells, curcumin-treated cells demonstrated reduced cell viability in the MTT assay at 24 hours (fig. 10). The CellTiter-Glo® Luminescent Cell Viability assay is a homogeneous method to determine the number of viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells. Curcumin led to a significant reduction in ATP production between the concentrations of 12.5 and 50 μM. Moreover, a dose-dependent decrease in cell viability between the concentration range of 12.5 and 35 μM was observed (fig. 11).
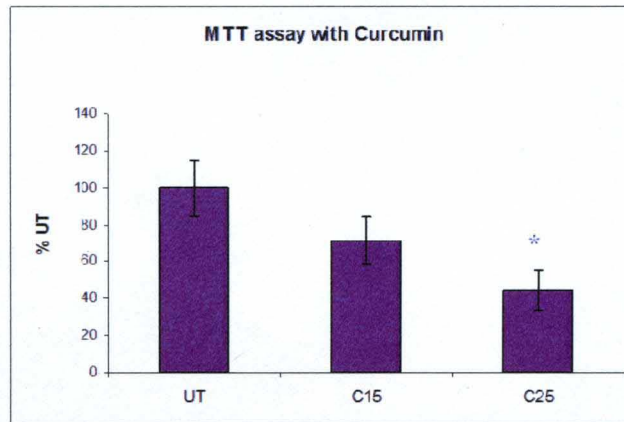
Fig.10 Curcumin displays cytotoxicity of HepG2 cells

Cells were left untreated (UT) or treated with curcumin (15 and 25 µM) for 24 hours and the MTT assay was performed. Data is presented as mean ± SD, n=3, * $p < 0.05$ when compared to UT
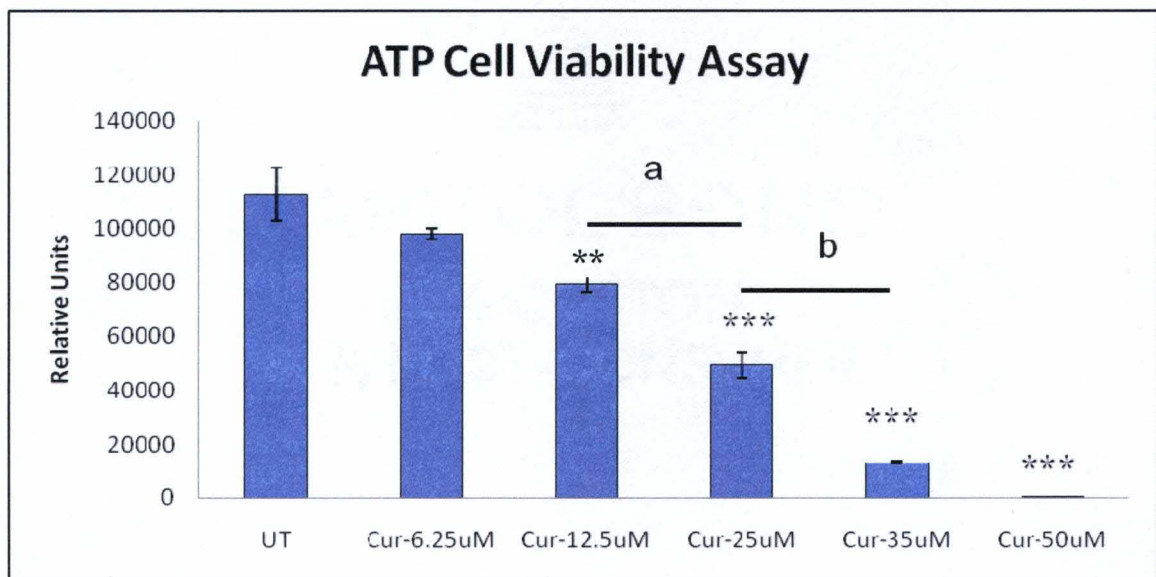

Fig.11 Curcumin reduces cell viability of HepG2 cells

Cells were left untreated (UT) or treated with curcumin (6.25 to 50 µM) for 24 hours and the CellTiter-Glo® Luminescent Cell Viability assay was performed. Data is presented as mean ± SEM, n=2; **$p < 0.01$, *** $p < 0.0001$ when compared to UT. a, b = $p < 0.01$

CHAPTER IV

SIGNIFICANCE AND CLINICAL RELEVANCE

All the current therapeutic options for the management of HCC have proven inadequate and do not offer satisfactory outcomes. There continues to be an acute need for effective therapies. Moreover, the need is getting increasingly bigger with the rapidly rising incidence of this highly lethal cancer. Combinatorial therapy is the cornerstone of most cancer therapy regimens today. Even in the case of HCC, combination therapy or adjuvant chemotherapy appears to be the most promising option for effective control. Adjuvant chemotherapy aimed at epigenetic modulation may afford even more benefit, given that epigenetic modifications play a role in HCC pathogenesis[33]. Curcumin has the potential to be an effective adjuvant chemotherapeutic agent, and could possibly enhance results of traditional chemotherapy through its unique mechanism of action. Along with present chemotherapies that mostly aim at tumor control by inhibition of DNA synthesis, derepression of critical silenced tumor suppressor genes, namely TFPI-2, by curcumin may provide more effective inhibition of tumor progression and metastasis. Adjuvant curcumin therapy will allow for reduction of dose of the more toxic anti-cancer drugs[33]. Moreover, as it is a natural compound with no side-effects or toxicity to normal cells, curcumin is not expected to cause

any toxic side-effects of its own. Several groups are currently working on the formulation of effective drug delivery systems for the administration of curcumin. In our future studies, we propose to use liposome encapsulated curcumin in an *in vivo* rat HCC model to improve the bioavailability of curcumin. With a well-elucidated mechanism of action in relation to TFPI-2 and an improved delivery system, curcumin could offer tremendous benefit to HCC patients as well as persons at risk for HCC development. The low cost and non-existent side-effect profile of curcumin only make it more promising as an ideal adjuvant chemotherapeutic drug.

CHAPTER V

FUTURE STUDIES

In further studies with curcumin and TFPI-2, we will investigate other epigenetic modifications that may be involved in curcumin-induced TFPI-2 reactivation. To this end, we will study histone H3 tri-methylation at lysine 4 (H3K4Me3), which denotes transcriptionally permissive chromatin. Histone 3 methylation at K9 and tri-methylation at K27, both of which represent transcriptionally repressive states, will also be examined. Also, DNA methylation at other sites in the TFPI-2 promoter will be examined using different primer sets. Binding of transcription factors and RNA polymerase II will also be studied. To assess the contribution of the reactivation of TFPI-2 to the anti-HCC effects of curcumin, HepG2 cells will be transfected with siRNA for TFPI-2 and cell survival and cell invasion assays will be performed.

The effects of curcumin on other tumor suppressor genes will also be examined in the future. The combinatorial effects of curcumin with current chemotherapeutic agents will be assessed, to study a potential adjuvant/combinatorial chemotherapeutic role for curcumin.

Finally, we propose to characterize the *in vivo effects* of curcumin using liposome-encapsulated curcumin in our rat xenograft model of HCC.

CHAPTER VI

SUMMARY

The goal of our study was to examine the anti-HCC effect of curcumin with respect to the derepression of the critical tumor suppressor gene, TFPI-2. Curcumin was observed to robustly reactivate TFPI-2 in HepG2 cells. TFPI-2 is epigenetically regulated in HCC cells. We therefore examined the epigenetic modifications at the TFPI-2 promoter region, specifically histone acetylation and DNA methylation as influenced by curcumin. Histone H3 acetylation at the transcription factor binding sites of the TFPI-2 promoter region was increased by curcumin and correlated with the induction of gene expression. This is the likely mechanism by which curcumin reactivates TFPI-2. The TFPI-2 promoter was found to be only slightly methylated at the single site investigated in this work and was not influenced by curcumin. However, other sites in the TFPI-2 promoter region need to be examined to fully characterize the effect of curcumin on CpG island methylation. Corresponding to the reactivation of TFPI-2, curcumin also induced cell death and reduced cell invasiveness in HepG2 cells. Further studies will examine the contribution of the epigenetic reactivation of TFPI-2 towards these anti-HCC effects.

Given the acute need for novel therapeutic options in the management of HCC, these studies have the potential to provide a basis for the development and use of curcumin in the clinical setting.

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AWARDS

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PUBLICATIONS

Shirish Barve, Roma Kapoor, **Akshata Moghe**, Julio A. Ramirez, John W. Eaton, Leila Gobejishvili, Swati Joshi-Barve and Craig J. McClain.
Alcohol Use, Highly Active Antiretroviral Therapy (HAART) and Liver Disease in HIV-Infected Patients. Alcohol Research and Health *In press*

Reactivation of the Epigenetically Silenced Tumor Suppressor Gene - Tissue Factor Pathway Inhibitor-2 (TFPI-2) by Curcumin Causes Cell Death in Hepatocellular Carcinoma Cells. **A. Moghe**, A.S. Barve, S. Ghare, L.Gobejishvili, S. Joshi-Barve, C.J. McClain and S. Barve.
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