RESEARCH ARTICLE

Curcumin Reorganizes miRNA Expression in a Mouse Model of Liver Fibrosis

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Abstract

Curcumin (CM), a biphenyl compound, possesses anti-inflammatory, antioxidant and antimicrobial activity. MicroRNAs (miRNAs) are small noncoding RNAs which regulate gene expression and the molecular mechanisms of several biological processes. Liver fibrosis is a major cause of hepatic dysfunction and cancer and there are few effective therapies emphasizing the need for new approaches to control. The present study was conducted to investigate the effect of curcumin (CM) on liver fibrosis through modulating the expression level of miRNAs (199 and 200), the main miRNAs associated with liver fibrosis. Induction of liver fibrosis by carbon tetrachloride (CCL_4) was confirmed by histopathological examination. Mice were divided into 3 groups: group 1 were i.p injected with 10% CCL_4 twice weekly for 4 weeks and then once a week for the next 4 weeks followed by 4 weeks with olive oil only. Group 2 were i.p injected with 10% CCL_4 twice weekly for 4 weeks and then once a week for the next 4 weeks followed by curcumin (5 mg/mouse/day) once daily for the next 4 weeks. The third group was injected with olive oil. The expression level of miR-199 and miR-200 and some of their targeted genes were measured by real time PCR. miRNA (199 and 200) levels were significantly elevated in liver fibrotic tissues compared to control groups. Curcumin was significantly returned the expression levels of mir-199 and -200 with their associated target gene nearly to their normal levels. This is the first study that highlighted the effect of curcumin on liver fibrosis through regulation of miRNAs.

Keywords: Curcumin - miRNAs - liver fibrosis - mouse model

Asian Pacific J Cancer Prev, **13 (11)**, 5405-5408

Introduction

Liver is the main organ involved in gluconeogenesis and in production and transportation of fatty acids and cholesterol in which its metabolic disease is prerequisited for cancer. Tissue fibrosis is a major cause of dysfunction in the hepatic systems (Glass et al., 2011). Hepatic fibrosis is influenced by several epigenetic factors that control the wound-healing response. Fibrosis is the excess deposition of extracellular matrix (ECM) components resulted from an imbalance of ECM molecule metabolism (Jiang et al., 2010; Glass et al., 2011) either by an increased synthesis or decreased degradation of ECM components or both. Collagens are synthesized by mesenchymal cells including fibroblasts and have an important role in the development of fibrosis (Jiang et al., 2010). MiRNAs are the smallest, non-coding RNA, plays important roles in post-transcriptional regulation (Borchert et al., 2006). In the nucleus, the miRNA genes are transcribed as primary-miRNA molecules, next processed forming pre-miRNAs (Lee et al., 2004; Borchert et al., 2006), finally transported to the cytoplasm for further processing (Lee et al., 2003; Lund et al., 2004). MiRNAs are associated with several pathophysiologic actions and their abnormal expression can associate with the liver diseases (Murakami et al. 2006; Jin et al., 2008; Ura et al., 2009; Yamamoto et al., 2009). Therefore, investigations have been conducted to discover the deregulation of miRNAs in diseases that may be of potential interest for therapeutic and basic medical research. The important changes in miRNA expression, acts in concert with other epigenetic factors, leads to fibrosis. Recent study has shown that miR-132 is significantly decreased in fibrotic livers as demonstrated in two different models (Murakami et al., 2011). MiRNAs, especially miRNA-199 and -200, are novel biomarkers for liver fibrosis and can regulate fibrosis (Murakami et al., 2011). MiR-29b, a negative regulator for the type I collagen, is a key regulator of liver fibrosis (Ogawa et al., 2009).

Curcumin (CM) presents in *Curcuma longa* herb as a biphenyl compound, possesses anti-inflammatory, antioxidant, wound healing and antimicrobial activities (Maheshwari et al., 2006) and has chemopreventive potential for several cancers (Perkins et al., 2002; Dorai et al., 2004; Choudhuri et al., 2005; Chen et al., 2006) by blocking steps in the carcinogenesis. CM acts on multiple targets and inhibits activation of key cell signaling mediators including NFκB, AP-1, Cox-2, MMP9 and EGFR (Chen et al., 2006; Shishodia et al., 2007).

Despite a difficult in understanding the fibrosis process, there are few effective therapies emphasizing the need for new therapies for fibrosis. This study was investigated

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the new effect of curcumin on liver fibrosis through modulation of the miRNA-199 and -200 expression patterns in induced liver fibrosis by using induced- liver fibrosis mouse model. This result might help in developing effective and safer therapy for liver fibrosis.

Materials and Methods

Chemicals and reagents

All chemicals used in this study were of analytical grade and products of Aldrich, Qiagene, Invitrogen and Jena Bioscience, Germany and England.

Animal experiments and chronic mouse liver injury model

This study was carried out in agreement with the recommendations in the Guide for the Care and Use of Laboratory Animals. The induction of fibrosis was done as previously described (Murakami et al., 2011). This study included three groups each with 15 adult male mice: Group 1 was given twice weekly an intra-peritoneal (i.p.) dose of a 10% CCL4 in olive oil (0.02 ml/g) for 4 weeks then once a week for the next 4 weeks followed by 4 weeks with olive oil only. Group 2 given twice weekly an i.p. dose of a 10% CCL4 in olive oil (0.02 ml/g) for 4 weeks then once a week for the next 4 weeks followed by CM (5 mg/mouse/day) once daily for the next 4 weeks. The third group was the control group and injected with olive oil only. At week 4, 8, and 12 the mice were sacrificed. Part of the livers were fixed, embedded in paraffin, and processed for histological confirmation of fibrosis and the other part were immediately stored at -70°C for RNA extraction.

Extraction and reverse transcription

The total RNA was isolated from 100 mg of frozen tissue with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Isolated RNA was analyzed and quantified on a Nano-Drop spectrophotometer. Total RNA (700 ug) was loaded to the micro RNA isolation column (Qiagene, Germany), for isolation of low molecular weight (LMW) RNA following the manufacturer's protocol. One microgram aliquot of DNase-treated total RNA and LMW RNA were reverse transcribed to cDNA using antisense of gene-specific primers, U6 and Thermoscript, thermostable reverse transcriptase (Invitrogen). One microgram RNA was incubated with 1.5 μl of a cocktail containing 10 mM each of the antisense gene specific and U6 primers. The reaction was denatured at 80^0C for 5 min, incubated for 5 min at 60^0C to anneal the primers, followed by cooling to room temperature and the remaining reagents [5 x buffers, dNTPs, DTT, RNase inhibitor, Thermoscript] were added as specified in the Thermoscript protocol and the reaction proceeded for 45 min at 60^0C. Finally, reverse transcriptase was inactivated by incubating the reaction at 85°C for 5 min. For each miRNA or gene all the reactions were run in triplicate and included no template and no reverse transcription as controls.

MiRNA expression by Real-Time PCR

The expression of the miRNA precursors was determined by using real-time quantitative PCR (Jiang et al., 2005). miRBase Sequences is the primary online repository for miRNA sequence data and annotation. miRBase Targets is a comprehensive new database of predicted miRNA target genes. miRBase is available at http://microrna.sanger.ac.uk/.Master mix contained 0.5 μl of 10x PCR buffer, 0.7 ul of 25 mM $MgCl_2$, 0.1μl of 12.5 mM dNTPs, 0.01 ul UNG, 0.5 μl of DNA Taq polymerase, 0.5 μl Eva Green (Jena Bioscience, Germany), 0.5 μl of dilute cDNA (1:50) and completed with water to 3 μl. Two microlitres from each primer was added to 3 μl master mix containing all of the reaction components. The reactions were amplified for 15s at 95^0C and 1 min at 60^0C for 40 cycles. At the end of the PCR dissociation curve was run to quantify the products copy number. To correct the systematic variables the data is commonly normalized to a universal endogenous control gene which is stably-expressed across the test sample set. The expression of each miRNA relative to U6 RNA was determined using the $2^{-\Delta CT}$ where ΔC_T=($C_T$miRNA-C_TU6RNA) relative gene expression multiplied by 10_5 .

Real Time PCR for genes expression

Real-time PCR was performed to detect the expression of fibrosis related genes. the primer sequences are as in Table 1. The expression levels of the target genes were normalized to β-actin gene expression as internal controls. The reactions were amplified for 15 s at 95^0C and 1 min at 60^0C for 40 cycles. At the end of the PCR dissociation curve was run to quantify the products copy number. The expression of each gene relative to β-actin was determined using the $2^{-\Delta CT}$.

Melting curve and agarose gel electrophoresis analysis

To verify the specificity of the product, melting curve analysis was performedand analysed using software of applied biosystem. Amplification plots and Tm values were analyzed to confirm the specificities of the amplicons for EvaGreen -based PCR amplification.

Validation of miRNA precursor primers by EvaGreen PCR

Each pair of primers included in this study was validated on extracted genomic DNA, mouse genomic DNA and no template control reaction.

Statistical analysis

Statistical analysis was performed using the SPSS version 9.0 software program and a statistical difference was considered significant when the p value <0.05 or equal.

Results

In order to identify the effect of curcumin on liver fibrosis through regulating the miRNA expression in the

Table 1. The Primers Sequences

Gene name	Sense	Antisense
MMP13	5'-gaggctccgagaaatgcagt-3'	5'-atgccatcgtgaagtctggt-3'
TIMP1	5'-cttggcttctgcactgatgg-3'	5'-acgctggtataaggtggtct-3'
a1-procollagen	5'-aacatgaccaaaaaccaaaagtg-3'	5'-cattgtttcctgtgtcttctgg-3'
β-actin	5'-ccactggcatcgtgatggac-3',	5'-tcattgccaatggtgatgacct-3'.

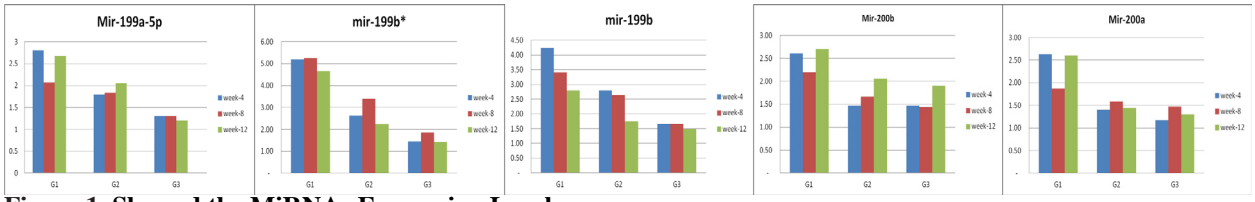

Figure 1. Showed the MiRNAs Expression Level

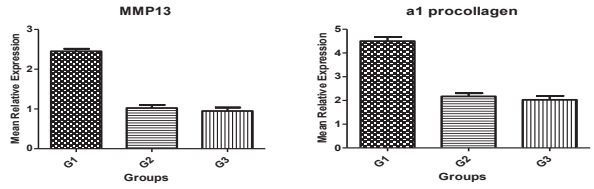

Figure 2. Showed the Gene Expression Level

treated mice with CM and the control groups (normal and fibrotic liver), we i.p. administered CCL4 in olive oil or olive oil for 12 weeks as in the study design for the three groups. Mice were sacrificed at 4, 8, or 12 weeks and then the degree of mouse liver fibrosis was determined by microscopy. Expression of mir-199 and -200 and their targeted genes were quantified by using real-time PCR.

A significant over-expression of miR-199a, 199a*, 200a and 200b was associated with the progression of liver fibrosis in group 1 compared to the control group. Also in the same group there was an upregulation in the a1-procollagen gene expression.

In order to reveal the effect of CM on the miR-199a, miR-199a*, miR-200a, and miR-200b, we studied the involvement of these miRNAs in the modulation of fibrosis-related gene. By studying the expression of fibrosis-related genes include a matrix degrading complex comprised of a1 procollagen, matrix remodeling complex include MMP13 and tissue inhibitors of metalloproteinases-1 (TIMP-1).

In addition, over-expression of miR-199a, miR-199a*, miR-200a and miR-200b was associated with significant upregulation in fibrosis-related genes, a1 procollagen, TIMP-1 compared with control group.

Finally in group 2 the expression levels of miR-199a and miR-199a* were nearly equal to the level of the normal group

Discussion

MicroRNAs are family of short non-coding RNAs that control normal rates of cellular growth, proliferation, differentiation and apoptosis, so their down-regulation may play a role in disease development (Croce, 2009). By targeting and controlling the expression of mRNA, miRNAs can control several biological pathways. The miRNAs associated with the liver fibrosis can regulate several fibrosis- related genes. In order to reveal the effect of CM on the miR-199a, miR-199a*, miR-200a, and miR-200b, we studied the involvement of these miRNAs in the modulation of fibrosis-related gene. By studying the expression of fibrosis-related genes include a matrix degrading complex comprised of a1 procollagen, matrix remodeling complex include MMP13 and tissue inhibitors of metalloproteinases-1 (TIMP-1). In addition, over-expression of miR-199a, miR-199a*, miR-200a and miR-200b was associated with significant upregulation in fibrosis-related genes, a1 procollagen, TIMP-1 compared with control group. In group 2 the expression levels of miR-199a and miR-199a* were nearly equal to the level of the normal group.

Recently, a study has investigated the miRNA regulation in a mouse model of CCl4-induced hepatic fibrogenesis with specification on those deregulated in livers during hepatic fibrosis. They found that the mir-29 family were significantly down-regulated in livers of CCL4-treated mice and was mediated by TGFβ, TLR signaling and activation of NF-ϰB signaling cascade (Roderburg et al., 2010). Also in human livers, they found lower expression of mir-29 in patients with advanced fibrosis (Roderburg et al., 2010). They concluded that the mir-29 mediates liver fibrosis regulation by modulating TGF-β1- and NF-ϰB-dependent. Similarly, other study reported many extracellular matrix (ECM) genes down-regulation by mir-29 and recommended the mir-29 as a potential therapeutic agent to treat liver fibrosis (Chau et al., 2011).

Recently, miR-23b was shown to play an important role in the termination of liver regeneration by activating TGF-β1 and Smad3 signalling in rats (Nagata et al., 2009) and the upregulation of miR-23b inhibited TGF-β1-driven apoptosis. Also, rno-miR-34 family was reported to be up-regulated in rats-induced hepatic fibrosis in (Li et al., 2011).The mir-199 and mir-200 families have are related to liver fibrosis. In the current study, a significant over-expression of miR-199a, 199a*, 200a and 200b was associated with liver fibrosis in group 1 compared to the control group. Also in the same group there was an upregulation in the a1-procollagen MMP13, and TIMP-1 genes expression. Similarly, in a study on human and murine miRNAs (miR-199a, antisense miR-199a*, miR-200a, and miR-200b) were significantly upregulated in progressing liver fibrosis in mice that were compared to controls in a CCL4-induced mouse model compared to olive oil-treated animals (Murakami et al., 2011). Experimental results were correlated with human data. Progression of hepatic fibrosis in this CCl4-driven model was shown to be linked to and significantly correlated with over-expression of the miR-199 and miR-200 (Murakami et al., 2011). Liver fibrogenesis is known to be mainly induced by abnormal expression of TGF-β1 (Yang and Mahato, 2011). TGFβ-induced factor (TGIF) plays role in the TGFβ signaling pathway, that are the targets of miR-199a* and miR-200b, respectively (Haybaeck et al., 2011). Down regulation of mir-199a, -199a* and -200a in chronic liver injury tissue was associated with the hepatocarcinogenesis (Murakami et al., 2006). In contrast to this study, others monitored the silence of mir-199a* expression in several proliferating cell lines excluding

fibroblasts (Kim et al., 2008).

In conclusion, tissue fibrosis is a major cause of organ dysfunction in diseases of the systems. This study increases the evidence that MiR-199 and miR-200 families are common regulator of fibrosis in the liver. Despite a difficult in understanding the fibrosis, emphasizing the need for treatment intervention. This is the first study that highlighted the effect of curcumin on liver fibrosis through the regulation of the miRNAs.

Acknowledgements

The authors extend their appreciation to the deanship of scientific reasearch at king Saud University for funding the work through the research group project No. RGP-VPP-074.

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