RESEARCH ARTICLE

Evaluation of MiR-34 Family and DNA Methyltransferases 1, 3A, 3B Gene Expression Levels in Hepatocellular Carcinoma Following Treatment with Dendrosomal Nanocurcumin

Fatemeh Chamani, Majid Sadeghizadeh*, Mahboubeh Masoumi, Sadegh Babashah

Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver making up more than 80 percent of cases. It is known to be the sixth most prevalent cancer and the third most frequent cause of cancer related death worldwide. Epigenetic regulation constitutes an important mechanism by which dietary components can selectively activate or inactivate target gene expression. The miR-34 family members including mir-34a, mir-34b and mir-34c are tumor suppressor micro RNAs, which are expressed in the majority of normal tissues. Several studies have indicated silencing of miR-34 expression via DNA methylation in multiple types of cancers. Bioactive nutrients like curcumin (Cur) have excellent anticarcinogenic activity and minimal toxic manifestations in biological systems. This compound has recently been determined to induce epigenetic changes. However, Cur is lipophilic and has a poor systemic bioavailability and poor absorption. Its bioavailability is increased through employing dendosome nanoparticles. The aim of the current study was to investigate the effect of dendrosomal nanocurcumin (DNC) on expression of mir-34 family members in two HCC cell lines, HepG2 and Huh7. We performed the MTT assay to evaluate DNC and dendosome effects on cell viability. The ability of DNC to alter expression of the mir-34 family and DNA methyltransferases (DNMT1, DNMT3A and 3B) was evaluated using semi-quantitative and quantitative PCR. We observed the entrance of DNC into HepG2 and Huh7 cells. Gene expression assays indicated that DNC treatment upregulated mir34a, mir34b and mir34c expression (P<0.05) as well as downregulated DNMT1, DNMT3A and DNMT3B expression (P<0.05) in both HepG2 and Huh7 cell lines. DNC also reduced viability of Huh7 and HepG2 cells through restoration of mir-34 expression. We showed that DNC could awaken the epigenetically silenced miR-34 family by downregulation of DNMTs. Our findings suggest that DNC has potential in epigenetic therapy of HCC.

Keywords: Hepatocellular carcinoma - MTT assay - dendrosomal nanocurcumin - microRNA-34 - DNA methyltransferase

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant cancer of the liver with high rates of lethality in the world (Lope et al; 2012). It is the fifth most common cancer worldwide and the third leading cause of death from cancer in the world, with resistance to chemotherapy and poor prognosis (Yao et al; 2009). It is as an epithelial tumor that is originated from stem cells or mature hepatocytes (Malenstein et al; 2011). The major risk factors of HCC are viral hepatitis infection, chronic alcohol consumption, fungal toxins (aflatoxins), nonalcoholic fatty liver disease (NAFLD), hemochromatosis and steroid hormone(Yang et al., 2010, Gaviñan et al., 2013). HCC occurs commonly and with increasing frequency in developing countries (85%) such as sub-Saharan Africa and Southeast Asia that viral hepatitis infection is epidemic (El-Serag 2011). Despite advances of treatment in recent years, HCC has a high resistance against conventional therapy. Therefore, new diagnostic and therapeutic approaches are urgently required. Discovery of genetic and epigenetic events in HCC development including genetic variations, histone modifications, DNA methylation and microRNAs have attracted great interest in this matter (Anestopoulos et al; 2015)

MicroRNAs (miRNAs) are a class of small non-coding RNAs with approximately 22–24 nucleotides in length and negatively regulate post translation of genes involved in cellular processes such as cell proliferation, apoptosis, invasion, metastasis, angiogenesis, differentiation, developmental timing and organ development (Lee et al;
Numerous approaches including the use of liposomes, nanoparticles, micelles, adjuvants and phospholipid complexes have been explored to improve the bioavailability of Cur (Anand et al.; 2007). Here, we employed dendrosome nanoparticles to increase the bioavailability and solubility of this agent in comparison with other carriers such as micelles, liposomes and phospholipids. Dendrosome nanoparticles, previously synthesized by our group (Sadeghizadeh et al; 2008) have several advantages such as stability, electrically neutral, biodegradability, spherical structure, non-toxic and easy preparation. Previous studies by our group have shown that dendrosomal nano curcumin (DNC) increase uptake of curcumin into cancer cells without any toxic effects on normal cells (Babaei et al., 2012, Tahmasebi Mirgani et al., 2014). Therefore, the aim of the current study is to test the gene expression levels of miR-34 family members and DNMTs in Huh7 and HepG2 cells following treatment with DNC.

Table 1. Primers Sequence Used for q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome Location</th>
<th>Primer</th>
<th>Sequence</th>
<th>Ampliqon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>19p13.2</td>
<td>Forward</td>
<td>5ʹ-GAAGGAGCCCGTGATG-3ʹ</td>
<td>233 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5ʹ-GTGTAGTGCTGTGATG-3ʹ</td>
<td></td>
</tr>
<tr>
<td>DNMT3A</td>
<td>2p23</td>
<td>Forward</td>
<td>5ʹ-TACGCAACACCTCAC-3ʹ</td>
<td>110 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5ʹ-AGATGTCTCAAGTGTC-3ʹ</td>
<td></td>
</tr>
<tr>
<td>DNMT3B</td>
<td>20q11.2</td>
<td>Forward</td>
<td>5ʹ-CGACCTACAGACGAC-3ʹ</td>
<td>170 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5ʹ-ATTGAATCTTCCAC-3ʹ</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>12p13</td>
<td>Forward</td>
<td>5ʹ-GTGAAACCTGAGAATGACAC-3ʹ</td>
<td>123 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5ʹ-CATGAGCTTCCAGGATACC-3</td>
<td></td>
</tr>
</tbody>
</table>

bp: base pair
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Semi quantitative reverse transcription PCR (sqRT-PCR)

After reverse transcription reaction in order to ensure the accuracy and specificity of the primers, reverse transcription PCR reaction was performed on the RT product. PCR was done using Maste Mix (Ampliqon, Denmark) at the following conditions: denaturation at 94 ° C for 30 seconds in a cycle and annealing at 60 ° C for 30 seconds in 35 cycles. PCR products were separated on 2% agarose gel electrophoresis and were observed by using ultraviolet rays after staining with ethidium bromide.

Real-Time quantitative reverse transcription-PCR (Real-Time qRT-PCR)

Real-time PCR was performed using the SYBR® Premix Ex Taq TM II (Takara Bio Inc., Shiga, Japan). The relative amount of DNMTs gene expression levels were normalized to the expression of GAPDH as house-keeping gene. Relative gene expression was calculated using the comparative cycle threshold (RQ=2 –ΔΔCT) method.

MiRNA expression analysis

RNA was extracted from either control or treated group using TRIzol® reagent (Life Technologies). Due to the small size of the microRNA, a poly A nucleotide tail was added to the miRNAs using polyA polymerase. In the reverse transcription step, cDNA is reverse transcribed using specific reverse primers and reverse transcriptase enzyme using MiR-Amp kit (Pars Genome, Tehran, Iran), according to the manufacturer’s instruction. In the PCR step, PCR products are amplified from cDNA samples using 5× HOT FIRE POL® EvaGreen® qPCR Mixplus ROX (Solis Bio Dyne, Tartu, Estonia) kit. The expressions

Table 2: IC50 of DNC for two hepatoma cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 for 24h</th>
<th>IC50 for 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huh7</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>HepG2</td>
<td>30</td>
<td>26</td>
</tr>
</tbody>
</table>

Figure 2. Cytotoxic Effects of DNC on Cancerous Cells. HepG2 and HuH7 Cells were Treated with Different Concentrations of DNC: (a) HuH7 (0–35 μM) for 24h, (b) HuH7 (0–30 μM) for 48h, (c) HepG2 (0–45 μM) for 24h, and (d) HepG2 (0–38 μM) for 48h were Checked for Their Proliferation by MTT Assay. DNC: Dendrosomal Nanocurcumin; IC50, 50 % Inhibitory Concentrations
of miR-34a, miR-34b, miR-34c and U6 (reference gene) were analyzed using universal and specific primer sets MiR-Amp kit (ParsGenome, Tehran, Iran). Relative gene expression was calculated using the comparative cycle threshold (RQ=2−ΔΔCT) method.

Statistical analysis
All statistical analyses were performed using Prism®5 software (Graph Pad Software, Inc., LaJolla, CA, USA) and Microsoft Excel. Data were presented in one way analysis of variance followed by Newman-Keuls multiple comparison test or Student’s t-test to compare expression between two group. The P<0.05 was considered statistically significant.

Results
Curcumin inhibit hepatoma cancer cell proliferation
The cytotoxic effect of DNC on HepG2 and HuH-7 cell lines could be seen microscopically as vesicular bodies in DNC treated cells in Figure 1 b and d compared to control ones in Fig.1 a and c. We also performed a MTT assay to study the effects of curcumin on the proliferation of HuH7 and HepG2 hepatoma cell lines. These cell lines were treated with multiple concentrations of curcumin for either 24h or 48h. As shown in Figure 2 a and b, 25µM and 15µM concentrations of DNC killed 50% of Huh7 cells after 24h and 48h, respectively. So IC50 values for DNC in Huh7 cells is 25µM for 24h and 15µM for treated 48 h. Furthermore, as shown in Fig.2 c and d, 30µM and 26µM concentrations of DNC killed 50% of HepG2 cells after 24h and 48h, respectively. So the IC50 value of DNC for HepG2 cell line with in 24h was about 30µM which relatively reduced to 26µM in 48h (Table2). Administration of DNC leaded to significant cell death in HuH7 and HepG2 cell lines, in a time and dose dependent manner.

DNC up-regulates miR-34a, miR-34b and miR-34c in hepatoma cell lines

DNC down-regulates DNMT1, DNMT3A and DNMT3B in Hepatoma Cell Lines .

Figure 3. DNC up-Regulates miR-34a, miR-34b and miR-34c in Hepatoma Cell Lines . a) miR-34a b) miR-34b and c) miR-34c in HepG2, d) miR-34a e) miR-34b and f) miR-34c in HuH7. Bars Represent Mean and Standard Error of Three Independent Experiments. Control: Non-Treated Sample, ns: Non Significant, *P<0.05, ** P<0.01.

Figure 4. DNC Down-Regulates DNMT1, DNMT3A and DNMT3B in Hepatoma Cell Lines . a) DNMT1 b) DNMT3A and c) DNMT3B in HepG2 , d) DNMT1 e)DNMT3A and f) DNMT3B in HuH7. Bars Represent Mean and Standard Error of Three Independent Experiments. Control: Non-Treated Sample, ns: Non Significant, *P<0.05,**P<0.01,***
Discussion

Bioactive nutrients including curcumin (a biphenyl compound derived from rhizome) offer great potential in causing cancerous cell death. In current study, using MTT assay, we indicated that HCC cells lines treated with DNC showed a time and dose dependent reduced viability compared with untreated cells. We used DNC with higher bioavailability compared to curcumin. The dendrosomes were shown to be safe for entrance of curcumin to cancer cells and only DNC leads to death of cancer cells and dendrosome has facilitated delivery of curcumin to cancer cells (Babaei et al; 2012, Tahmasebi Mirgani et al; 2014, Zamani et al; 2015).

The miR-34 family members including mir34a, mir34b and mir34c acts as tumor suppressor microRNAs. Hence, their reduction or loss of expression was reported in a large number of tumors including breast, colorectal, melanoma and HCC (Gopalan et al; 2014, Li et al; 2014). However, no studies have been performed to determine whether miR-34 family could be re-expressed in hepatoma cancer by DNC. We found that DNC upregulated mir34a, mir34b and mir34c expression (P<0.05) in both HepG2 and Huh7 cell lines. We indicated that these upregulation is the result of DNMTs down-regulation.

Further more, our findings demonstrate that the expression of DNMT1, DNMT3A and DNMT3B is greatly down-regulated under treated with DNC, suggesting that DNC restores the expression of mir-34 family members via down-regulation of these enzymes. These findings suggest that DNC potentially could be used in clinic especially for HCC therapy.

Conflict of interest
None of the authors have any conflicts of interest in this work.

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References


