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ORIGINAL PAPER

Induction of cancer-related microRNA expression profiling using excretory-secretory products of *Clonorchis sinensis*

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Abstract *Clonorchis sinensis* is a carcinogenic human liver fluke by which chronic infection is strongly associated with the development of cholangiocarcinoma. Although this cholangiocarcinoma is caused by both physical and chemical irritation from direct contact with adult worms and their excretory–secretory products (ESPs), the precise molecular events of the host–pathogen interactions remain to be elucidated. To better understand the effect of *C. sinensis* infection on cholangiocarcinogenesis, we profiled the kinetics of changes in cancer-related microRNAs (miRNAs) in human cholangiocarcinoma cells (HuCCT1) treated with *C. sinensis* ESPs for different periods. Using miRNA microarray chips containing 135 cancer-related miRNAs, we identified 16 miRNAs showing differentially altered expression following ESP exposure. Of these miRNAs, 13 were upregulated and 3 were downregulated in a time-dependent manner compared with untreated controls. Functional clustering of these dysregulated miRNAs revealed involvement in cell proliferation, inflammation, oncogene activation/suppression, migration/invasion/metastasis, and DNA methylation. In particular, decreased expression of let-7i, a tumor suppressor miRNA, was found to be associated with the ESP-induced upregulation of TLR4 mRNA and protein, which contribute to host immune responses against liver fluke infection. Further real-time quantitative PCR analysis using ESP-treated normal cholangiocytes (H69) revealed that the expressions of nine miRNAs (miR-16-2, miR-93, miR-95, miR-153, miR-195, miR-199-3P, let7a, let7i, and miR-124a) were similarly regulated, indicating that the cell proliferation and inhibition of tumor suppression mediated by these miRNAs is common to both cancerous and non-cancerous cells. These findings constitute further our understanding of the multiple cholangiocarcinogenic pathways triggered by liver fluke infection.

Keywords *Clonorchis sinensis* · Cholangiocarcinoma · MicroRNA array · Excretory–secretory products

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Introduction

Cholangiocarcinoma (CCA) is an aggressive malignancy of the bile duct epithelium that is classified as intrahepatic, extrahepatic, or perihilar according to its anatomical distribution. Because CCA is diagnosed at advanced stages, it is considered an incurable and highly lethal cancer with poor survival outcomes unless the primary tumor and any metastases can be completely resected. The delayed diagnosis is due to the absence of specific symptoms and the lack of precise screening systems for early or premalignant stage disease (Blechacz and Gores 2008).

Known risk factors for CCA include primary sclerosing cholangitis, liver fluke infestation, exposure to nitrosamine, and choledochal cysts, but most patients have no identifiable specific risk factors. Compared with Europe or North America, there is a much higher incidence of CCA in Southeast Asian countries where infection with liver flukes such as *Clonorchis sinensis* and *Opisthorchis viverrini* is common due to more habitual ingestion of raw or

undercooked freshwater fish (Rustagi and Dasanu 2012). Since both experimental and epidemiological studies have provided sufficient evidence for a correlation between liver fluke infection and CCA, the International Agency for Research on Cancer (IRAC) has recently classified liver fluke as a group I biological human carcinogen (Bouvard et al. 2009).

Although the precise molecular mechanisms of carcinogenesis associated with liver fluke infestation are not fully understood, it has been hypothesized that persistent injury and inflammation of the biliary epithelia and surrounding liver tissue resulting from both mechanical and chemical irritation contribute to carcinogenesis through chronic liver fluke infection. Mechanical damage is caused by physical contact with the worms during their feeding and migratory activities while chemical damage is caused by their excretory–secretory products (ESPs). These two types of damage together lead to hyperplasia and adenomatous changes in the bile duct epithelia with subsequent malignant transformation of cholangiocytes into CCA (Sripa et al. 2012). In particular, cells exposed to ESPs from liver flukes display diverse pathophysiological responses, including proliferation, apoptosis, and inflammation (Thuwajit et al. 2004; Kim et al. 2008; Serradell et al. 2007; Nam et al. 2012). We previously profiled the changes in the transcriptomes and proteomes of human CCA cells (HuCCT1) exposed to *C. sinensis* ESPs (Pak et al. 2009a, b). The genes/proteins identified participate in apoptotic modulation, carcinogenesis, metabolism, signal transduction, and redox homeostasis, implying that ESP plays a pivotal role in modification of the host cell state. In addition, treatment with *C. sinensis* ESPs enhances acetylation of histone H3 and 4 in HuCCT1 cells via activation of histone acetyltransferases, suggesting the involvement of ESPs in chromatin remodeling (Kim et al. 2010).

MicroRNAs (miRNAs) are a class of small non-coding RNAs, typically 20–25 nucleotides in length, which inhibit gene expression by either degrading target mRNA or suppressing translation by binding to the 3′-untranslated region of the target mRNA. They participate in the modulation of various physiological pathways, including development, differentiation, apoptosis, morphogenesis, and metabolism. Systematic expression analysis of miRNA profiles has revealed that numerous miRNAs are upregulated or downregulated in multiple tumors compared with normal tissues, indicating a possible relationship between miRNA and oncogenesis (Esquela-Kerscher and Slack 2006; Zhang et al. 2007). Indeed, it is generally accepted that miRNAs function as both tumor suppressors and oncogenes, playing roles in the networking of tumor progression and metastasis processes. For example, the expression of the oncomer miR-21 is inversely correlated with that of the tumor suppressor let-7a during tumorigenesis of liver fluke-associated CCA in an animal model and in human CCA samples (Namwat et al. 2012). In addition, the expression of DNA methyltransferase-1 is modulated in a miRNA-dependent manner in interleukin-6-overexpressing malignant cholangiocytes, resulting in upregulation of methylation-sensitive tumor suppressor genes such as Rassf1a and p16INK4a (Braconi et al. 2010).

To investigate whether a carcinogenic expression signature of miRNAs is associated with liver fluke infestation, we assessed the time course of the differential expression of 135 cancer-related miRNAs (using miRNA microarray chips) in HuCCT1 cells treated with *C. sinensis* ESPs. Using real-time quantitative PCR (qPCR), we also examined the expression patterns of these differentially regulated miRNAs in ESP-treated normal cholangiocyte cells (H69 cells). Our current study may provide a basis for further exploration of the functional roles of miRNAs in the development, progression, diagnosis, and prognosis of liver fluke-associated CCA.

Materials and methods

Materials

Cell culture medium components were purchased from Life Technologies (Grand Island, NY) unless otherwise indicated. The human H69 cholangiocyte cell line was kindly provided by Dr. Dae Ghon Kim of the Department of Internal Medicine, Chonbuk National University Medical School, Jeonju, Korea. The ESPs were prepared as described previously (Nam et al. 2012), aliquoted, and stored at −80 °C until use. All other chemicals (biotechnology grade) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and ESP treatment

The human HuCCT1 CCA cell line was maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and an antibiotic mixture. H69 cells between passages 25 and 30 were grown in Dulbecco's Modified Eagle Medium (DMEM)/F12 (1:1) containing 10 % FBS, an antibiotic mixture, 1.8×10^{-4} M adenine, 5 μg/ml insulin, 5.5×10^{-6} M epinephrine, 2×10^{-9} M triiodothyronine, 5 μg/ml transferrin, 1.64×10^{-6} M epidermal growth factor (EGF), and 1.1×10^{-6} M hydrocortisone. Both cell types were cultured at 37 °C in a humidified 5 % CO_2 incubator. For ESP treatment, cells were seeded at ~70 % confluence in six-well culture dishes and grown for 24 h under normal culture conditions. Cells were gradually deprived of serum by incubation in 1 % FBS overnight. After being incubated for an additional 3 h in serum-free medium, cells were treated with 800 ng/ml ESPs and incubated for 1–24 h.

Total RNA preparation

Cells exposed to ESPs were harvested at each time point, and total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA) and further purified using RNeasy columns (Qiagen, Valencia, CA) according to the manufacturers' instructions. After DNase digestion and clean-up procedures, the RNA samples were quantified and stored at −80 °C until use.

miRNA microarray

Differential miRNA expression profiling was performed using a peptide nucleic acid-miRNA expression profiling kit (Panagene Inc., Daejeon, Korea) according to the manufacturer's protocols. Briefly, denatured total RNA (400 ng) was hybridized to a miRNA microarray slide containing 135 cancer-related miRNA probes for 4 h at 55 °C. After extensive washing, the bound mature miRNA was labeled with pCp-Cy3 through a T4 RNA ligase reaction for 2 h in a 37 °C humidified atmosphere. With termination of labeling by washing, hybridized arrays were scanned with an Axon GenePix 4000B scanner (Molecular Devices Corp., Sunnyvale, CA) and median spot intensities were determined using Axon GenePix 4.0 (Molecular Devices Corp.). Further data analysis was performed using Microsoft Excel. Selected miRNAs were visualized by using a heatmap and dChip software analyzer.

Analyses of miRNA using real-time qPCR

Differentially regulated miRNAs were further examined in H69 cells under the same ESP treatment conditions detailed above. A small RNA-rich fraction was isolated using a miRNeasy Mini kit (Qiagen), followed by synthesis of first-strand cDNA using a miScript Reverse Transcription Kit (Qiagen) in accordance with the manufacturer's instructions. qPCR was performed in a LightCycler 480 system (Roche Diagnostics Inc., Basel, Switzerland) using a miScript SYBR-Green PCR kit (Qiagen). The amplification reaction was performed under the following conditions: 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s with miScript Universal Primer (Qiagen), the forward primers of each miRNA, and small nuclear RNA (RNU6B) as an internal control. The primer sequences for each miRNA are detailed in Table 1. The miRNA expression levels were normalized after subtracting the cycle threshold (C_t) value of the RNU6B internal control from that of each miRNA C_t value for each time point sample. The relative level of miRNA at each ESP-treated time point was compared with that of the untreated control (0 h) by setting the miRNA expression in the control to 1 and determining the fold change in expression against this value, calculated as $2^{-\Delta\Delta Ct}$.

Table 1 Primer sequences of mature miRNAs for qPCR

miRNAs	Mature miRNA sequences: 5′–3′
let-7a	CUAUACAAUCUACUGUCUUUC
let-7i	UGAGGUAGUAGUUUGUGCUGUU
miR-16	CCAAUAUUACUGUGCUGCUUUA
miR-24	UGGCUCAGUUCAGCAGGAACAG
miR-31	AGGCAAGAUGCUGGCAUAGCU
miR-93	ACUGCUGAGCUAGCACUUCCCG
miR-95	UUCAACGGGUAUUUAUUGAGCA
miR-124a	UAAGGCACGCGGUGAAUGCC
miR-136	CAUCAUCGUCUCAAAUGAGUCU
miR-153	UUGCAUAGUCACAAAAGUGAUC
miR-181d	AACAUUCAUUGUUGUCGGUGGGU
miR-185	AGGGGCUGGCUUUCCUCUGGUC
miR-195	CCAAUAUUGGCUGUGCUGCUCC
miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA
miR-342-5p	AGGGGUGCUAUCUGUGAUUGA
miR-373	ACUCAAAAUGGGGGCGCUUUCC

Semi-quantitative reverse transcription-PCR

Approximately 1 μg of total RNA was applied to synthesize first-strand cDNA with amfiRivert cDNA Synthesis Master Mix (GenDeport, Barker, TX), followed by amplification with Taq polymerase (ExTaq; TaKaRa Bio, Inc., Shiga, Japan). The primer sequences were as follows: human toll-like receptor 4 (TLR4) cDNA, 5′-CTGCAATGGATCAAGGACCA-3′ (forward) and 5′-TCCCACTCCAGCTAAGTGTT-3′ (reverse), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, 5′-ACCCAGAAGA CTGTGGATGG-3′ (forward) and 5′-CAGGAAATGAGC TTGACAAAG-3′ (reverse). The thermocycling conditions were as follows: 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s. PCR products were separated on a 1.2 % agarose gel, and images were analyzed for the quantitation of DNA band densities with a Fluor-S MultiImager (Bio-Rad, Hercules, CA).

Immunoblotting

Total soluble proteins from ESP-treated HuCCT1 cells were extracted using RIPA buffer with a complete protease inhibitor cocktail (Sigma-Aldrich), and the protein concentration was determined using a DC protein assay (Bio-Rad). Thirty micrograms of total soluble proteins were separated by SDS-PAGE on 12 % gels and transferred onto nitrocellulose membranes (GE Healthcare Biosciences, Uppsala, Sweden). The membranes were probed with primary antibody to TLR4 (Abcam, Cambridge, UK), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody

(Jackson ImmunoResearch Laboratory, West Grove, PA). The immune complexes were detected with enhanced chemiluminescence (GE Healthcare Biosciences) and quantitated by densitometric scanning of the X-ray film with a Fluor-S MultiImager (Bio-Rad). Blots were normalized for protein loading by washing in BlotFresh Western Blot Stripping Reagent (SignaGen Laboratories, Gaithersburg, MD) and reprobing with a polyclonal antibody to GAPDH (AbFrontier Co., Seoul, Korea).

Statistical analyses

Data are expressed as means±standard error of three or more independent experiments. Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by a Student's *t* test or Bonferroni's test, as appropriate. Differences in mean values were considered statistically significant at $p<0.05$.

Results and discussion

Expression profiles of cancer-related miRNAs in ESP-treated HuCCT1 cells

We previously profiled differentially expressed genes in ESP-treated HuCCT1 cells using cDNA microarrays containing human genes of known function. The identified upregulated genes were involved in oncogenesis or cell proliferation/differentiation, whereas the downregulated genes participated in apoptosis, suggesting that ESPs might induce carcinogenic signal transduction pathways (Pak et al. 2009a). This finding prompted us to further examine changes in the expression of cancer-related miRNAs in response to ESPs.

To identify differentially expressed miRNAs, PANArray™ miRNA microarray slides were hybridized with total RNA samples obtained after 0, 1, 3, 9, 15, and 24 h of incubation of HuCCT1 cells with ESPs and then labeled. Two independent experiments allowed selection of those miRNAs showing good reproducibility and reliability with an alteration tendency (a >1.2- or <0.8-fold change in expression between each time point and the untreated control). Among 135 cancer-related miRNAs, we identified 16 miRNAs whose expression levels were upregulated (13 miRNAs) or downregulated (3 miRNAs) in ESP-treated cells compared with the untreated (0 h) control (Fig. 1). Out of the 13 upregulated miRNAs, 7 (miR-16-2, miR-24, miR-31, miR-93, miR-153, miR-185, and miR-199a-3p) showed gradually increased expression patterns in proportion to ESP exposure time, and the expressions of 4 (miR-95, miR-136, miR-195, and miR-373) were increased at early (1–3 h) and late (15–24 h) time points after ESP treatment. In addition, the expression of miR-181d was significantly increased between 3 and 9 h, followed by gradual decreases afterward, and the expression of miR-342-5p was drastically increased at only 24 h of ESP treatment (Fig. 1a). Meanwhile, there were three miRNAs (let-7a, let-7i, and miR-124a) for which the expression gradually declined in inverse proportion to the exposure time (Fig. 1b).

Based on the function of their putative target genes, the differentially regulated miRNAs were classified into biological groups (Fig. 1c). Many of these miRNAs are involved in cell proliferation (44 %; 7/16), followed by the main modulators for inflammation (32 %; 5/16). In addition, 2 of the 16 miRNAs act as oncogenic miRNAs (oncomirs) that promote cancer formation and/or inhibit the activation of tumor suppressors (Galihouste et al. 2013). One other identified miRNA is associated with DNA methylation, which is an important factor in carcinogenic mechanisms, while another was found to be linked to tumor migration, invasion, and metastasis, respectively. These functional results for the regulated miRNA clusters were consistent with the progressive symptoms caused by liver fluke infection, such as inflammation, fibrosis, and even the development of CCA (Sripa et al. 2012).

Analysis of upregulated miRNAs

Among our 13 upregulated miRNAs (Fig. 1a), 7 were mainly involved in proliferation, namely, miR-16-2, miR-93, miR-95, miR-136, miR-153, miR-195, and miR-199a-3p. The expression of most of these miRNAs gradually increased in ESP-treated cells in a time-dependent manner. It has been reported that the expression of miR-16-2, miR-93, and miR-95 is upregulated in each case in esophageal adenocarcinoma, breast cancer, and colorectal carcinoma tissues, respectively, and that the overexpression of these molecules in the respective cells increases tumor cell proliferation and metastasis (Hu et al. 2011; Fang et al. 2012; Huang et al. 2011). In a mouse breast carcinoma cell line, miR-136 was found to target the tumor suppressor PTEN, with a consequent tumor-promoting role in cancer development (Lee et al. 2010). Moreover, the suppression of miR-136 led to reduced growth of human lung cancer cells via the inhibition of Erk1/2 phosphorylation through direct targeting of the Ser/Thr protein phosphatase 2A 55-kDa regulatory subunit B α isoform (Shen et al. 2014). PTEN is also a direct target of miR-153, and its overexpression promotes proliferation of prostate cancer cells via increased expression of G1/S translational promoter and cyclin D1 with a concomitant decrease in p21 expression (Wu et al. 2013).

Overexpression of miR-199a-3p increases the proliferative and survival activities of human breast carcinoma cells, accompanied by an inhibition of caveolin-2 expression (Shatseva et al. 2011). We observed increased expression of miR-195, which was previously shown to suppress growth by inducing G1 arrest in hepatocellular carcinoma cells (Furuta

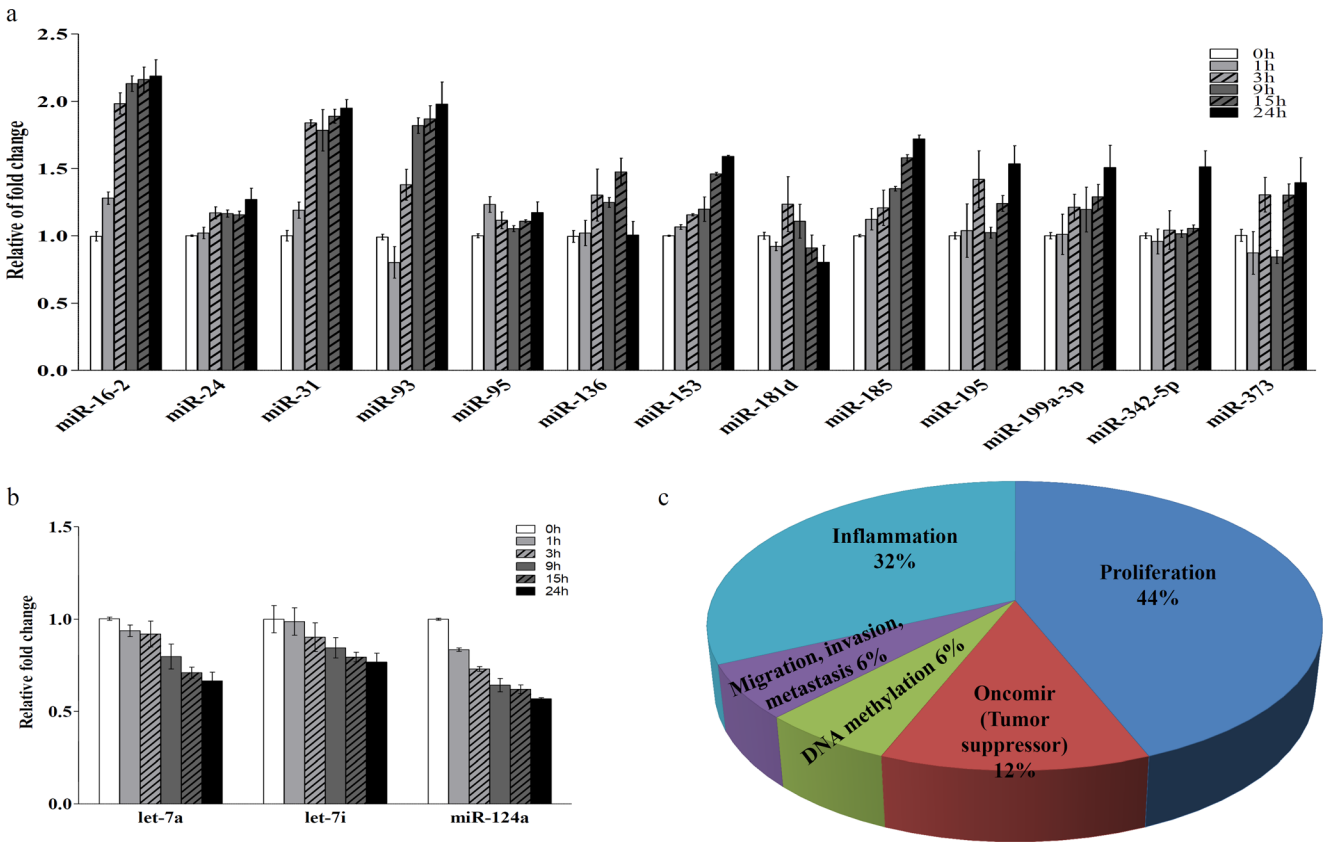

Fig. 1 Differentially regulated cancer-related miRNAs in response to *C. sinensis* ESPs. HuCCT1 cells were treated with 800 ng/ml ESPs, harvested between 0 and 24 h later, and subjected to miRNA microarray analysis. **a** Upregulated miRNA expression profile. **b** Downregulated miRNA expression profile. **c** Functional classification of differentially expressed miRNAs according to their target gene functions. The data are presented as fold change of all of the individual identified miRNAs

et al. 2013). Detailed analyses of miRNA clusters on chromosomes and of cross-signaling between cell cycle regulators targeted by each miRNA will help to elucidate the mechanism of this complementary miRNA overexpression. Our current observation of an induction of miRNAs involved in proliferation is consistent with the previous finding that ESPs from *C. sinensis* and *O. viverrini* stimulate epithelial cell proliferation by inducing transcription factor and cell cycle regulatory proteins (Thuwajit et al. 2004; Kim et al. 2008).

In our current experiments, ESP treatment increased the expression levels of miR-31 and miR-185, miRNAs that are ubiquitously expressed in normal tissues but are highly enriched in tumors. A relatively high expression of miR-31 has been observed in cancerous versus normal tissues from mouse and human lungs (Liu et al. 2010a, b) and in samples from patients with hepatocellular carcinoma and intrahepatic CCA (Karakatsanis et al. 2013). The expression level of miR-185 is higher in clear-cell renal cell carcinoma than in adjacent normal kidney tissue obtained from the same patients (Liu et al. 2010a, b). These earlier studies further demonstrate that both miR-31 and miR-185 function as oncomirs in the respective cancer types by targeting specific tumor suppressors for repression; large tumor suppressor 2 (LAST2) and PP2A regulatory subunit B α isoforms (PPP2R2A) are repressed by miR-31, whereas PTEN or Fas-associated protein tyrosine phosphatase and putative tumor suppressor (PTPN13) are anti-correlated with miR-185. Hence, ESPs may participate in liver fluke-induced cholangiocarcinogenesis by inhibiting cancer prevention pathways.

Aberrant genomic DNA methylation patterns (hypomethylation in repetitive DNA regions and hypermethylation in gene promoter regions) play an important role in tumorigenesis. In particular, inactivation of tumor suppressor genes via hypermethylation of their promoter regions is closely associated with human malignancies (Ozdemir et al. 2012). Upregulation of miR-181d was detected in malignant ovarian cancer tissues and directly targeted CDH13 (H-cadherin) and RASSF1, which are tumor suppressor genes inactivated by hypermethylation (Lee et al. 2012). In glioblastoma, miR-181d bound to the 3′ UTR of the methylguanine methyltransferase transcript, resulting in repression of DNA repair activity following temozolomide-induced DNA damage (Zhang et al. 2012). ESPs induced the upregulation of miR-181d in our current study, along with the global histone hyperacetylation status, in ESP-treated CCA cells as reported previously (Kim et al. 2010), suggesting that ESPs contribute to epigenetic alterations during liver fluke infestation.

The levels of miR-24 and miR-373 expressions were increased in ESP-treated cells in our present analyses. These miRNAs are characterized as oncomirs that modulate the expression of several proteins related to cell adhesion, migration, invasion, and metastasis. For example, ectopic miR-24 expression in breast cancer cells and tumors activates epidermal growth factor receptor via the repression of tyrosine protein phosphatase activities. Furthermore, the expression levels of several matrix metalloproteinase (MMP) isozymes are upregulated, resulting in enhanced tumor growth, tumor local invasion, and metastasis (Du et al. 2013). It has been reported that miR-373 upregulates MMP9 expression by activating the Ras/Raf/MEK/Erk signaling pathway and NF-κB via the blockage of mTOR and SIRT1 translation, thus promoting the migration and metastasis of human fibrosarcoma cells (Liu and Wilson 2011). In an *O. viverrini*-infected CCA animal model, increased expression of MMP9 in the liver is time-dependently correlated with myofibroblast accumulation, fibrosis levels, and cholangiocarcinogenesis (Prakobwong et al. 2010). We have found previously that treatment of microfluidic three-dimensional cultured CCA cells with gradient *C. sinensis* ESPs induces MMP isozyme expression with a concomitant increase in focal and adhesion molecules, promoting aggregation and invasion into neighboring extracellular matrix (Won et al., manuscript in preparation). Taken together, the evidence indicates that dysregulation of MMPs via miR-24 and/or miR-373 may be a crucial factor in cancer cell migration, invasion, and metastasis in liver fluke-induced CCA.

Our ESP treatments resulted in increased expression of miR-342-5p at a late exposure time (24 h). The analysis of the miRNA real-time PCR array revealed that the expression of macrophage-derived miR-342-5p is upregulated in early atherosclerotic lesions and induces the activation of proinflammatory mediators by directly inhibiting Akt1, demonstrating a crucial role of miR-342-5p in the early inflammatory response in lesional macrophages (Wei et al. 2013). This inflammation-associated miRNA induction is consistent with our previous finding that enzymatic production of free radicals in *C. sinensis* ESP-treated CCA cells causes NF-κB-mediated inflammation (Nam et al. 2012). Since chronic inflammation of infected bile ducts is a known predisposing factor in the pathogenesis of CCA induced by liver fluke, the miR-342-5p/inflammation link may provide a potential therapeutic target for the treatment of inflammation-associated cancer.

Analysis of downregulated miRNAs

The expression levels of three miRNAs (let-7a, let-7i, and miR-124) known as tumor suppressors gradually declined in response

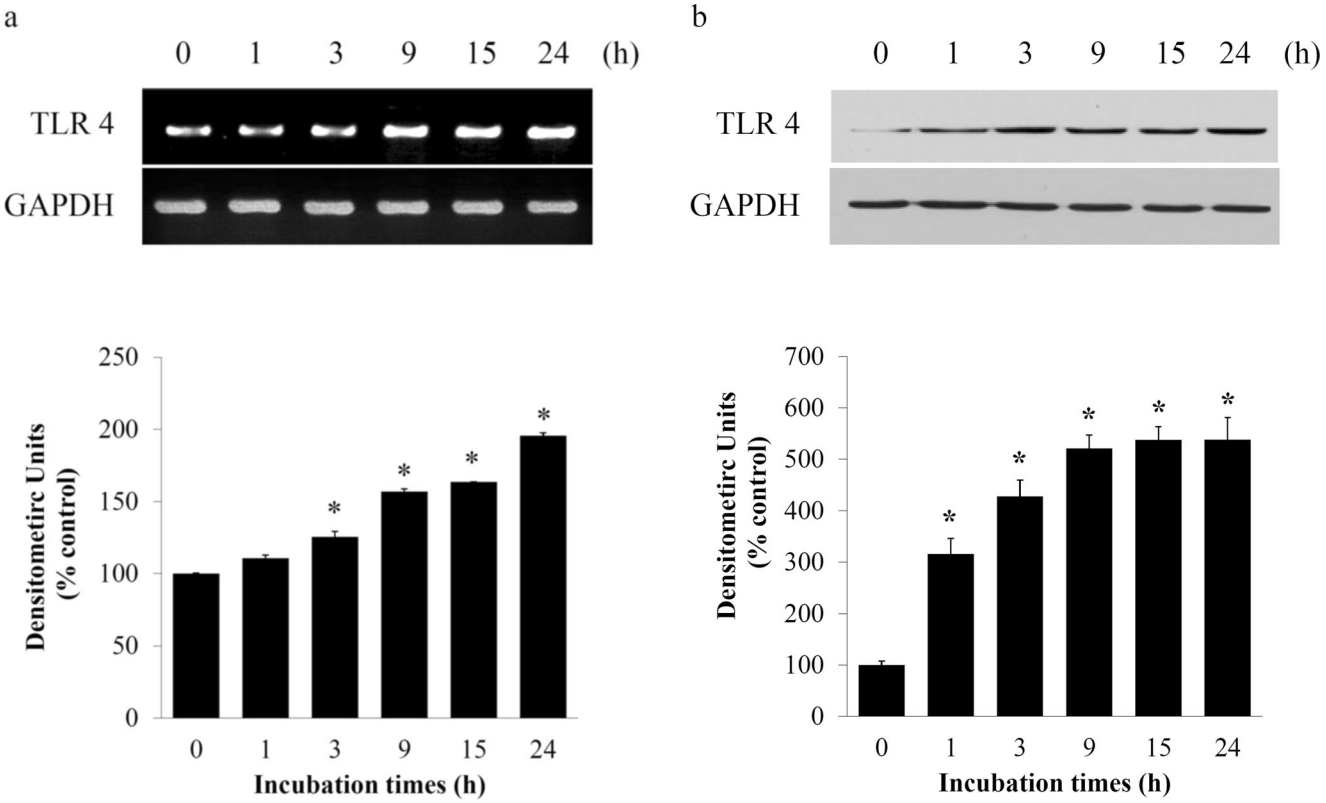

Fig. 2 Effect of ESPs on TLR4 mRNA and protein expression. HuCCT1 cells were treated with 800 ng/ml ESPs, harvested between 0 and 24 h later, and subjected to expression analysis. **a** Semi-quantitative RT-PCR of TLR4 mRNA. **b** Representative immunoblot of TLR4. Individual data were quantified as densitometric units and normalized to GAPDH mRNA and protein. *Data in the graphs* are shown as a percentage relative to zero time and presented as means±standard error for three independent experiments. *$p<0.05$, compared with zero time

to ESP exposure (Fig. 1b). Let-7 is the prototype of a miRNA family that is highly conserved from invertebrates to humans. It represses multiple oncogenes, such as RAS, MYC, and HMGA2. Thus, its dysregulation affects the proliferation and differentiation of tumor cells (Bussing et al. 2008). The expression level of let-7a is downregulated during the genesis of *Opisthorchiasis*-associated CCA in both an animal model and human surgical samples (Namwat et al. 2012). Microbial infection (*Cryptosporidium parvum*) of human cholangiocytes (H69) decreases let-7i expression via a MyD88/NF-κB-dependent mechanism, which is associated with upregulation of TLR4 expression in infected cells. This result indicates that altered let-7i expression plays an essential role in epithelial immune responses against *C. parvum* infection (Chen et al. 2007). Consistent with the results of that study, we found in our current analyses that TLR4 mRNA and protein levels were increased in ESP-treated HuCCT1 cells in a time-dependent manner (Fig. 2), suggesting that reciprocal expression of let-7i and TLR4 influence host cell regulatory responses against liver fluke infection, such as the NF-κB-induced inflammation signaling pathway.

The tumor-suppressive activity of miR-124 has been reported in hepatocellular carcinoma and gastric cancer, and its expression is downregulated in the respective cell lines and tumor tissues (Lu et al. 2013; Xie et al. 2014). Overexpression of miR-124 in HepG-2 cells inhibits cell proliferation, induces apoptosis, and suppresses tumor growth in vivo by directly targeting STAT3. In gastric cancer cells, the same suppressive effects of miR-124 on proliferation and tumor growth are achieved by restoring its expression, which directly attenuates the expression of EZH2 protein. Therefore, it is persuasive to postulate that the downregulation of miR-124 partly contributes to the liver fluke-associated induction of cell proliferation during the development of CCA.

Expression of differentially regulated miRNAs in ESP-treated cholangiocytes

To elucidate whether ESP-mediated miRNA dysregulation in cancer cells is a common phenomenon, we used qPCR to analyze the expression patterns of our identified 16 miRNAs in a normal human cholangiocyte cell line (H69) exposed to ESPs for between 0 and 24 h. Among the 13 miRNAs upregulated in HuCCT1 cells, the expression of 6 (miR-16-2, miR-93, miR-95, miR-153, miR-195, and miR-199a-3p) was

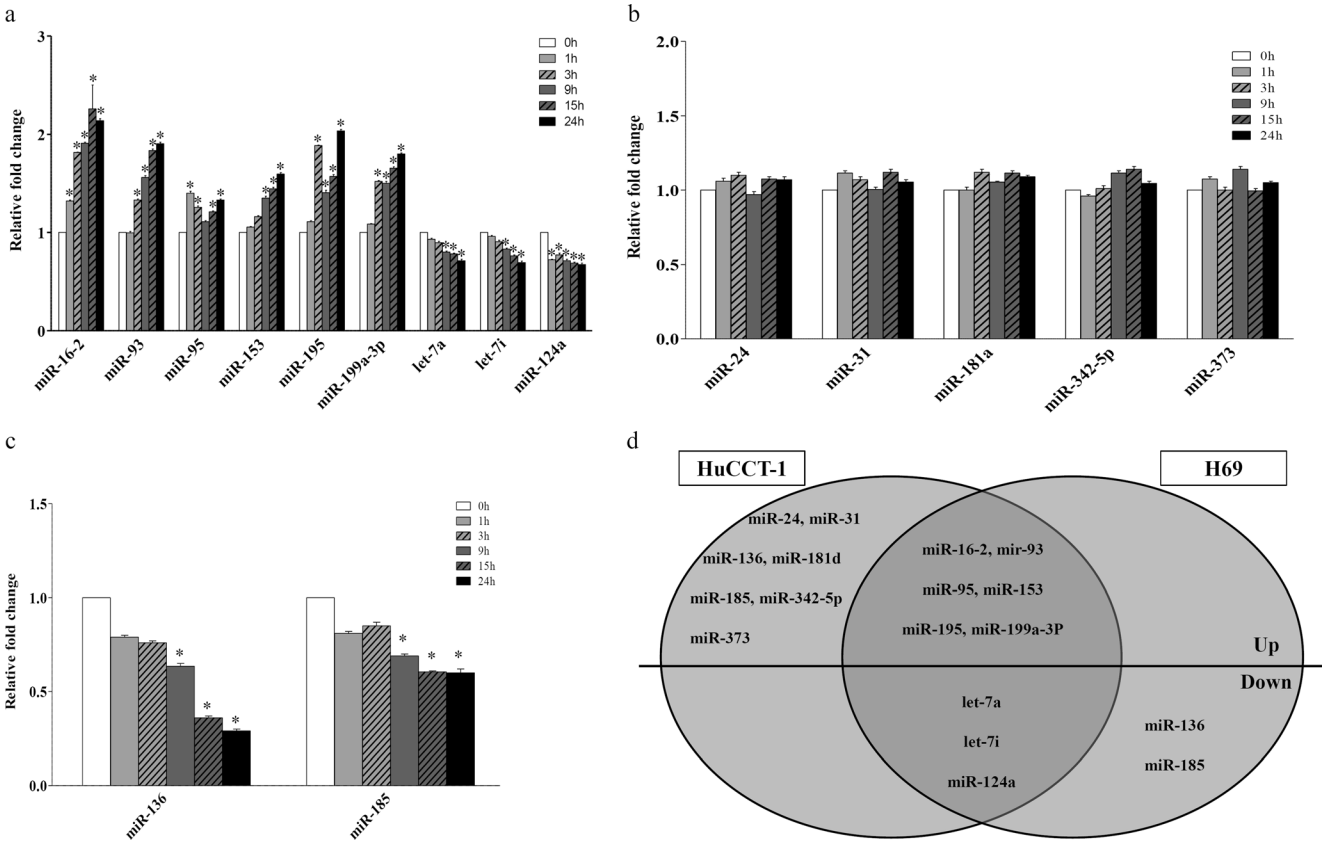

Fig. 3 Expression patterns of regulated miRNAs in ESP-treated normal cholangiocytes (H69 cells). Normal cholangiocytes (H69 cells) were treated with 800 ng/ml ESPs for 0 to 24 h and then harvested for qPCR analysis. The miRNA level at each time point was calculated as the fold change ($2^{-\Delta\Delta Ct}$) relative to the untreated control (0 h) after normalization to small nuclear RNA (RNU6B). The *error bars* represent $2^{-\Delta\Delta Ct}\pm$ standard error of three independent experiments. **a** Similarly upregulated miRNA expression. **b** Unchanged miRNA expression. **c** Oppositely downregulated miRNA expression. **d** Venn diagram showing similarly and differently regulated miRNAs between HuCCT1 and H69 cells

increased in ESP-treated H69 cells. In addition, the expression of three miRNAs (let-7i, let7a, and miR-124a) downregulated in HuCCT1 cells was also decreased in H69 cells (Fig. 3a). These results suggest that common regulatory mechanisms for these miRNAs occur in both cancerous and non-cancerous bile duct epithelial cells in response to ESPs. Upregulated and downregulated miRNAs in both ESP-treated cells linked to cell proliferation and tumor suppression, respectively, which coincide with the effect of liver fluke ESPs on cell proliferation (Thuwajit et al. 2004; Kim et al. 2008).

The expression levels of five miRNAs (miR-24, miR-31, miR-181d, miR-342-5p, and miR-373) upregulated in ESP-treated HuCCT1 cells were unaltered in H69 cells (Fig. 3b). These miRNAs are involved in tumor progression activities, such as DNA methylation, migration, invasion, inflammation, and downregulation of tumor suppressor genes. Moreover, the expression of two upregulated miRNAs (miR-136 and miR-185) in HuCCT1 cells was decreased in ESP-treated H69 cells (Fig. 3c). These differences may be due to differential gene expression/regulation between cancer and normal cells, cell type-dependent effects of liver fluke ESPs, and/or multitarget gene properties of miRNAs.

In conclusion, we have performed cancer-related miRNA profiling analysis using miRNA microarrays of host cells treated with *C. sinensis* ESPs, an approach that mimics carcinogenic liver fluke infection in vitro. To our knowledge, our current study represents the first attempt to describe global changes in cancer-related miRNA expression in response to liver fluke ESPs. Information obtained from this analysis enabled us to identify potential target genes involved in multiple oncogenic pathways during the infection. In addition, the distinctive dysregulation patterns of miRNA expression will yield further insight into the classification of different stages of liver fluke-associated tumor progression and provide promising curative targets for molecular mechanism-based approaches.

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Conflict of interest The authors declare that they have no conflict of interest.

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