Role of miR-18a and miR-25 disruption and its mechanistic pattern in progression of liver cancer

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Received: 15 December 2019 / Accepted: 8 January 2020 / Published online: 28 January 2020
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Abstract

This study examined the molecular mechanisms underlying the roles of the microRNAs miR-18a and miR-25 in the progression of human liver cancer. Liver cancer biopsies obtained from early-stage liver cancer patients were examined by qRT-PCR and Northern blotting to examine the expression of miR-18a and miR-25. Both microRNAs were overexpressed in mouse primary hepatocytes following transfection of the cells with vectors encoding the microRNAs. An analysis of biopsy samples from liver cancer patients indicated that both miR-18a and miR-25 were overexpressed during the early stages of liver cancer. Further, qRT-PCR and Northern blotting confirmed that both of these microRNAs play crucial roles in the progression of liver cancer. Our findings clearly indicate that miR-18a and miR-25 can be used as prognostic biomarkers for early-stage liver cancer. Hence, miR-18a and miR-25 may have value as prognostic indicators and may facilitate the development of novel therapeutics for liver cancer.

Keywords Liver cancer · miR-18a · miR-25 · Northern blotting · qRT-PCR

Introduction

The liver plays a number of crucial roles in the human body (Gao et al. 2008). For example, the liver is involved in digestion, detoxification, metabolism, and nutrient storage (Farazi and DePinho 2006). A number of disease states due to viral infection and alcohol consumption are known to be mediated by liver injury (Farazi and DePinho 2006). Liver cancer is one of the most commonly diagnosed types of cancer, ranking as the fifth most common cancer worldwide (Bosch et al. 2004). Men are more susceptible than women to liver cancer, which is the third largest cause of cancer mortality (Bruix et al. 2004).

MicroRNAs are small non-coding RNAs 19–27 nucleotides in length that play important roles in cell differentiation, proliferation, and apoptosis (MenDell 2005). Briefly,

microRNAs bind to the 3'-untranslated region (UTR) of their target mRNAs and induce the degradation or inhibit the translation of these mRNAs, thereby modifying the expression of their target genes, thus, they are involved in the progression of diseases. Recent studies suggested the deregulation of microRNAs in hepatocellular carcinoma (HCC), specifically those related to clinicopathological features such as metastasis, recurrence, and prognosis (Braconi and Patel 2008; Ladeiro et al. 2008; Mott 2009). Transcription factors that are enriched in the liver (e.g., HNF1A, HNF3A, and HNF3B) act as regulatory molecules involved in the loss of miR-122 in HCC resulting in increase in cell migration and invasion (Coulouarn et al. 2009). Gramantieri et al. (2007) demonstrated that miR-122 targets cyclin G1 expression and thereby suppresses HCC cell growth. Interestingly, miR-221 and miR-222 function as oncogenes in HCC by binding to target sites in the 3'-UTR of p27 (le Sage et al. 2007). In addition to liver cancer, microRNAs have been reported to be involved in cardiovascular diseases as well as other types of cancer. The microRNA miR-29 was shown to inhibit collagen biosynthesis and to be involved in myocardial infarction and the progression of cardiac fibrosis (Van Rooij et al. 2008). By targeting the Smad4 gene, miR-34a regulates the fibrosis that occurs after myocardial infarction, and elevated

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miR-24 expression was shown to reduce smooth muscle cell function (Huang et al. 2014; Fiedler et al. 2014).

The expression levels of two microRNAs, miR-25 and miR-18a, were shown to be upregulated in HCC (Braconi et al. 2011). In addition, many other microRNAs are either upregulated or downregulated in HCC (Braconi et al. 2011). Interestingly, miR-18a, which is overexpressed in liver cancer, has been suggested to be related to the 4.5:1 female to male ratio for liver cancer (Liu et al. 2009). Overexpression of miR-18a downregulates expression of the ESR1 gene, which encodes estrogen receptor (ER) alpha, thereby resulting in an increased risk of liver cancer in women (Liu et al. 2009). miR-18a belongs to the miR-17-92 cluster, and hence targets p21, which is a gatekeeper of the G1/S checkpoint. Similarly, miR-25 also targets p21, thereby altering the cell cycle and leading to apoptosis. It is important to study both microRNAs and identify their mechanistic roles in the context of liver cancer. The present study examined the mechanisms underlying the effects of miR-18a and miR-25 disruption in the progression of liver cancer.

Materials and methods

Patients and samples

Liver cancer biopsy specimens were collected from 18 patients (11 males and 7 females) at affiliated hospitals with appropriate patient consent and ethics committee approval (Ref. No. DFT20180179). The biopsy materials were frozen and stored in liquid nitrogen immediately after surgery. As the biopsies were from different types of patients, histological examination was performed to determine the stages of liver cancer and the tumors were graded according to World Health Organization (WHO) standards. Of the 18 samples, 11 were classified as early-stage liver cancer and the remaining 7 were classified as advanced-stage liver cancer. The mean $\pm$ SE age of the total study population was 31.4 ± 11.7 years, while those of the patients with early-stage and advanced-stage liver cancer was 43.8 ± 6.2 years and 54.9 ± 13.9 years, respectively.

RNA extraction

RNA was isolated from 18 liver cancer biopsy specimens using the TRIzol method (Invitrogen) in accordance with the manufacturer's protocol. The quality and concentration of RNA were analyzed using a NanoDrop spectrophotometer (Thermo Scientific), and the reliability of the RNAs was evaluated by agarose gel electrophoresis. The RNA samples were then used for qRT-PCR and Northern blotting.

qRT-PCR analyses of miR-18a and miR-25

To examine the expression of miR-18a and miR-25, qRT-PCR was performed using a MirVana qRT-PCR miRNA Detection Kit. RT-PCR was performed for miR-18a and miR-25 (primer catalog numbers: 30115, 30119, and 30302; Ambion) (Guang-Hui Liu et al. 2013). RNA samples were used as templates for cDNA synthesis at 37 °C for 30 min using reverse transcriptase. Quantitative RT-PCR was performed to examine the expression of miR-18a and miR-25 along with appropriate controls. The expression levels of miR-18a and miR-25 were quantified using the Δ Ct method. The proportion of expression was estimated using the equation $2^{-\Delta\text{CT}}$, where $\Delta\text{CT} = [\text{CT}(\text{miR-221/miR-222}) - \text{CT}(5S \text{ rRNA})]$ (Morimura et al. 2011).

Northern blotting analysis of miR-18a and miR-25

Northern blotting was performed to confirm the expression of miR-18a and miR-25 in liver cancer biopsy samples. RNA was extracted from liver cancer biopsy samples using TRIzol reagent (Invitrogen), run on 12% denaturing polyacrylamide gels, and then transferred to nylon membranes (Ambion). Hybridization of the membranes was carried out using digoxigenin (DIG)-labeled miR-18a and miR-25 probes (Sigma). The membranes were kept overnight in washing buffer ($5 \times \text{SSC}$, 20 mmol/L Na_2HPO_4 , pH 7.2, 7% SDS, $1 \times \text{Denhardt's}$ solution, and 0.2 mg/mL of salmon sperm DNA). The following probes were used for hybridization: miR-18a, 5'-GUCAACAUCAGUCUGAUAAGCUA-3'; miR-25, 5'-GUCAACAUCAGUCUGAUAAGCUA-3'; and U6 RNA as a loading control, 5'-GUC AACAUCAGUCUGAUAAGCUA-3'. Following incubation, the membranes were washed in a buffer containing $1 \times \text{SSC}/1\%$ SDS at 50 °C. After washing, the blots were examined using a DIG Luminescent Detection Kit (Roche) and analyzed by GeneGenius.

Cell culture and transfection of miR-18a and miR-25

Briefly, mouse primary hepatocytes (ATCC CRL-2254 cells) were cultured using DMEM/F12 medium supplemented with antibiotics. All hepatocyte cell culture media and supplements were purchased from Sigma-Aldrich. A mouse microRNA expression plasmid carrying GFP as a reporter for monitoring of transfection and a neomycin resistance selection marker was used to overexpress miR-18a and miR-25. The transfection of miR-18a and miR-25 was performed using LipofectAMINE 3000 Reagent

(Thermo Fisher). The transfection efficiency was determined by monitoring GFP expression.

Statistical analysis

The experiments were performed in triplicate and the results were analyzed using SPSS 11.5 software. All measurements are shown as the mean $\pm$ SE. In all analyses using an ANOVA and the χ^2 test, $P < 0.05$ was taken to indicate statistical significance.

Results

Collection of liver cancer samples and microRNA analysis

Liver cancer samples were collected by biopsy and the samples were graded based on the malignancy and WHO criteria. After grading, miR-18a and miR-25 expression

was examined in 11 early-stage liver cancer samples. Of 18 samples, 11 were from male patients and 7 samples were from female patients. The biopsy samples were obtained from an affiliated hospital with the approval of our internal institutional approval committee and ethics committee. Information related to the types of tumors and their grades is listed in Table 1. The samples were stored in liquid nitrogen immediately after collection until further study. The samples were then subjected to qRT-PCR and Northern blotting to examine the expression of miR-18a and miR-25 in early-stage liver cancer.

qRT-PCR analysis of miR-18a and miR-25 in liver cancer samples

miR-18a and miR-25 expression was determined by qRT-PCR analysis. The results indicate a considerable positive correlation between miR-18a and miR-25 expression in early-stage liver cancer (Fig. 1). The expression of miR-18a was detected in all samples, but especially high

Table 1 Liver cancer staging (BCLC)

Sl. no.	Stages	Patient samples (<i>n</i> = 18)	Tumor characteristics
Liver cancer staging (BCLC)			
1	Very early stage	1	Single tumor < 2 cm
2	Early Stage	11	Single tumor < 3 cm
3	Intermediate stage	4	Large multinodular
4	Advanced stage	2	Vascular invasion or extrahepatic spread
5	Terminal stage	—	Any of the above-mentioned tumor characters

Total number of patient samples (*n*) = 18; of the 18 samples 11 samples are from males; and 8 samples are from females

Fig. 1 Relative expression pattern of miR-18a and miR-25 in early, late and metastatic stage of liver cancer. **a** qPCR analysis of overexpression pattern of miR-18a during early, late and metastatic stage of

liver cancer patient samples. **b** qPCR analysis of overexpression pattern of miR-25 during early, late and metastatic stage of liver cancer patient samples

levels of expression were noted in early-stage liver cancer (Fig. 1). Our qRT-PCR findings for miR-25 were similar to those for miR-18a, indicating especially high levels of expression in early-stage liver cancer samples compared with late-stage liver cancer samples (Fig. 1).

Fig. 2 Northern blotting analysis of early, late and metastatic stage of liver cancer patient samples expressing miR-18a and miR-25. U6 RNA act as a control for the experiments

Northern blotting

To confirm the levels of miR-18a and miR-25 in early-stage liver cancer, we performed Northern blotting using the liver cancer biopsy samples (Fig. 2). The early-stage liver cancer biopsy samples were subjected to 12% denaturing polyacrylamide gel electrophoresis along with appropriate controls. The results showed positive and abundant expression of miR-18a and miR-25 in the liver cancer biopsy samples, especially in the samples from patients with early-stage liver cancer. The expression levels of miR-18a and miR-25 detected by Northern blotting in the late-stage liver cancer samples were lower than those in the early-stage liver cancer samples (Fig. 2). U6 RNA was used as a loading control. The data indicate that both miR-18a and miR-25 were over-expressed in the early-stage liver cancer biopsy samples. The intensity of the Northern blot was correlated with the control as well as the early and late-stage liver cancer biopsy

Fig. 3 Transfection of miR-18a, miR-25 and GFP in mice primary hepatocytes cells. **a** Normal mice primary hepatocytes cells at 40× magnification. **b** Mice primary hepatocytes cells transfected with

miR-18a and miR-25 at 40× magnification. **c** Expression pattern of miR-18a, miR-25 and GFP in transfected mice primary hepatocytes cells. Scale bar—100 μm

samples (Fig. 3). The upregulation of miR-18a was correlated with that of miR-25 in early-stage liver cancer.

Cell culture and transfection of miR-18a and miR-25

Mouse primary hepatocytes were purchased from ATCC and used for miR-18a and miR-25 overexpression studies. Briefly, normal mouse hepatocytes were transfected with expression vectors for miR-18a and miR-25. Transfection was confirmed using a GFP transfection assay kit. After transfection, the cells were subjected to qRT-PCR analysis to confirm the overexpression of miR-18a and miR-25. Mouse primary hepatocytes transfected with the miR-18a and miR-25 expression vectors were similar in morphology to HepG2 liver cancer cells. In addition, the miR-18a- and miR-25-transfected mouse primary hepatocytes showed expression of liver cancer markers (Fig. 3). The results outlined above indicate that the overexpression of miR-18a and miR-25 plays a role in the progression of liver cancer.

Discussion

Liver cancer is the fifth most common cancer worldwide (Yang et al. 2008). Due to its high mortality rate, it is important to understand the molecular mechanisms underlying its development to allow early diagnosis as well as for the development of possible treatment strategies. Some cancers, especially HCC, have high mortality rates in the early stages of disease progression (Heindryckx et al. 2009). Therefore, this study was performed to examine the expression pattern of microRNAs specifically upregulated in the early stages of liver cancer.

Our qRT-PCR results demonstrate the overexpression of miR-18a in early-stage liver cancer biopsy samples, suggesting that miR-18a is useful as a marker for the early diagnosis of liver cancer. To validate our qRT-PCR data, we performed Northern blotting, which clearly indicated that both miR-18a and miR-25 were overexpressed in the early stages of liver cancer (Fig. 3). Interestingly, gastric cancer cell lines were reported to show the downregulation of miR-18a, miR-21, and miR-221 (Mohammadian et al. 2016). Our data indicate the upregulation of miR-18a in the early stages of liver cancer. Taken together, our observations and the findings of previous studies suggest that alterations in miR-18a expression may be specific to each type of cancer, showing upregulation in the early stages of liver cancer but downregulation in gastric cancer cell lines. Interestingly, only miR-18a, and not miR-25, was downregulated in gastric cancer cell lines, suggesting that the upregulation and downregulation of microRNA expression are specific to different types of cancer.

To further confirm these results, we transfected mouse primary hepatocytes with expression vectors for both

miR-18a and miR-25 using LipofectAMINE. We used mouse primary hepatocytes cells in this study due to the ethical difficulties associated with the collection of normal hepatocytes from human subjects. The data shown in Fig. 3 clearly demonstrate the expression of liver cancer markers in cells overexpressing the microRNAs. After transfection with miR-18a or miR-25, the morphology of the mouse primary hepatocytes became similar to that of Hep G2 liver cancer cells. These observations suggest that overexpression of miR-18a and miR-25 resulted in the induction of liver cancer. Consistent with our data, previous reports clearly showed that miR-18a induced progression of nasopharyngeal carcinoma by impairing microRNA biogenesis (Luo et al. 2013). Interestingly, our study indicates the roles of miR-18a and miR-25 in the progression of liver cancer. Our findings indicate that both miR-18a and miR-25 may be useful as prognostic markers for liver cancer in the early stages. Additional studies are essential to explore the use of miR-18a and miR-25 in determining the prognosis of early liver cancer. Serum miR-18a was reported to be a potential marker for screening as well as prognosis of hepatitis B-dependent HCC (Li et al. 2012). Previous reports suggested that microRNAs are useful for determining the prognosis of cancer at the initial stages. Serum miR-21 and miR-92a have been used as biomarkers for the diagnosis of colorectal cancer (Liu et al. 2013). Similarly, circulating miR-18a in plasma has been used to detect pancreatic cancer (Morimura et al. 2011). Therefore, miR-18a and miR-25 may be useful as markers for screening as well as to predict the prognosis of liver cancer.

Conclusions

Our findings suggest that miR-18a and miR-25 may be useful as biomarkers for predicting the prognosis of liver cancer in the early stages. The expression levels of both microRNAs were upregulated in liver cancer patients. Hence, miR-18a and miR-25 may be useful as prognostic indicators and may facilitate the development of novel therapeutic strategies for liver cancer in the early stages of disease progression.

Acknowledgements The authors thank the funding agency for providing funds to complete the project. Also, the authors thank the patients for providing the consent for ethical clearance. Finally, authors thank the institution for providing infrastructure and instrumentation facility to perform the research.

Author contributions YL and ZM contributed equally to this manuscript by sharing the experiments. AQ and JW helped in sample collection from patients and preparation of RNA samples. XJ performed the Northern blotting experiments. ZQ is the corresponding author of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest in any part of the manuscript.

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