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Circulating microRNAs, possible indicators of progress of rat hepatocarcinogenesis from early stages

Tokuo Sukata, Kayo Sumida*, Masahiko Kushida, Keiko Ogata, Kaori Miyata, Setsuko Yabushita, Satoshi Uwagawa

Environmental Health Science Laboratory, Sumitomo Chemical Co., Ltd., 1-98, 3-Chome, Kasugade-Naka, Konohana-ku, Osaka 554-8558, Japan

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ABSTRACT

MicroRNAs (miRNAs), a class of small noncoding RNAs that regulate gene expression at the posttranscriptional level, are believed promising biomarkers for several diseases as well as a novel target of drugs, including cancer. In particular, miRNAs might allow detection of early stages of carcinogenesis. The present study was conducted to provide concrete evidence using chemical-induced hepatocarcinogenesis in rat as a model. We thereby observed aberrant fluctuation of circulating miRNAs in the serum of rats not only with neoplastic lesions such as hepatocellular adenoma (HCA) and hepatocellular carcinoma (HCC), but also with preneoplastic lesions, such as foci of hepatocellular alteration (FHA). Additional qRT-PCR analysis revealed gradual elevation of some circulating miRNAs (i.e., let-7a, let-7f, miR-34a, miR-98, miR-331, miR-338 and miR-652) with progress of hepatocarcinogenesis. Interestingly, increased levels of let-7a, let-7f and miR-98 were statistically significant even in the serum of rats at very early stages. These findings provide the first evidences that circulating miRNAs have the potential to predict carcinogenesis at earlier stages, preneoplastic lesions than with previous biomarkers and that they might be utilized to monitor the progress of tumor development.

1. Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs (~22 nucleotides) that have crucial roles under physiological and pathological conditions, such as development, metabolism, cellular differentiation, proliferation, cell-cycle control and cell death (Ambros, 2004; Zamore and Haley, 2005; Meister, 2007; Miska, 2005; Jannot and Simard, 2006). In addition, many studies have implicated miRNAs in genesis of a variety of human diseases, including cancer (Umbach et al., 2008; Thum et al., 2008; Calin et al., 2004), and reported an attractive aspect that the fluctuation of some specific circulating miRNAs in biofluid reflects various physiological changes and diseases in the past few years. Mitchell et al. (2008) demonstrated the presence of circulating tumor-derived miRNAs in blood through the research using a mouse prostate cancer xenograft model, suggesting the fluctuation of circulating miRNAs in blood is probably related to the lesion directly. Chen et al. (2008) demonstrated unique fluctuations of miRNAs dependent on kinds of neoplasms, like lung and colorectal cancers. Several other studies have also indicated diagnostic and prognostic utility (Lawrie et al., 2008; Resnick et al., 2009; Wong et al., 2008; Skog et al., 2008; Ng et al., 2009; Chim et al., 2008; Gilad et al., 2008; Taylor and Gercel-Taylor, 2008; J. Wang et al., 2009; K. Wang et al., 2009). However, there has hitherto been no clear evidence of any potential to detect early stages of disease, e.g. preneoplasia.

With respect to the hepatocellular carcinoma (HCC), which is one of the most common malignant tumors in humans and inducible by many chemicals, miRNAs are definitely related to carcinogenesis and tumor progression. Loss of miR-122 has been shown with metastasis (Coulouarn et al., 2009) and down-regulation of let-7c occurs in PPARα-induced hepatocarcinogenesis (Shah et al., 2007). In addition, Yamamoto et al. (2009) demonstrated an elevated level of miR-500 in sera from HCC patients and return to normal after surgical treatment. In the present study, we therefore explored the profiles of circulating miRNAs during rat hepatocarcinogenesis using sera from rats hepatic lesions induced by different types of carcinogenic compounds. Constitutive androstane receptor (CAR)-mediated hepatocarcinogenesis using phenobarbital and DDT, and peroxisome proliferator-activated receptor α (PPARα)-mediated hepatocarcinogenesis using clofibrate (Köhle et al., 2008) were selected as models. Thereby, we could demonstrate elevation of some specific miRNAs of not only in the sera of HCA- or HCC-bearing rats, but also animals with preneoplastic lesions. Interestingly, gradual increase was noted with progression of hepatocarcinogenesis, suggesting that circulating miRNAs are novel biomarkers predictive of even early stages of car-

* Corresponding author. Tel.: +81 6 6466 5306; fax: +81 6 6466 5319.
E-mail address: sumida@sc.sumitomo-chem.co.jp (K. Sumida).

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cinogenesis and might be utilized to monitor the process of tumor development.

2. Materials and methods

2.1. Animals and chemicals

N-nitrosodiethylamine (DEN), 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) and clofibrate (>98%) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and phenobarbital sodium (PB) (>98%) was purchased from Wako pure chemical industries (Osaka, Japan). Synthetic miR-3 from *Drosophila melanogaster* (dme-miR-3) was obtained from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). A total of 50 male 5-week old F344 rats were purchased from Charles River, Japan Inc. (Atsugi, Japan) and housed in suspended aluminum cages (three rats in a cage) in a room kept at 24 ± 2 °C temperature and 40–70% humidity with a 12-h light/dark cycle. They received CRF-1 Laboratory Chow (Charles River Japan, Inc.) as basal diet. The animals were observed daily and were used after a 1-week acclimation period for the experiments. Body weights were measured every week.

2.2. Animals and treatments

All experiments were performed in accordance with the Guide for Animal Care and Use of Sumitomo Chemical Co. Ltd. Animal treatment with some chemicals was conducted 6 years ago as follows. At the age of 6 weeks, 50 male F344 rats were divided into 5 groups (10 animals per group). Animals in Group 1 were injected with saline instead of DEN solution without subsequent administration of any chemicals. Animals in Group 2 were injected with DEN (100 mg/kg body weight), afterward, received the basal diet. Animals in Groups 3–5 were injected with DEN (100 mg/kg body weight) intraperitonealy once a week for 2 weeks, and after a one-week recovery period, received PB (500 ppm, Group 3), DDT (500 ppm, Group 4), or clofibrate (3000 ppm, Group 5) in diet, respectively. Animals from 5 animals (lowest identification numbers) were sacrificed at Week 26 in the each group for the examination of an early stage of carcinogenesis, and the remaining animals were sacrificed at Week 36 for the examination of the late stage. At the sacrifice, whole blood samples were withdrawn from the abdominal aorta of the all animals under ether anesthesia, and the liver tissues were also obtained from the all animals and treated with some appropriate procedures for the following examinations. The whole blood samples were separated into serum and cellular fractions after leaving at the room temperature for 30 min. Then the sera and liver tissues were stored at −80 °C.

2.3. Histopathological examination and laser microdissection (LMD)

Formalin-fixed paraffin-embedded liver tissues were sectioned at about 4 μm to get several sets of 7 serial sections, and the first and last sections were applied to routine HE staining for histopathological examination. The remaining sections were applied to casual HE in accordance with the protocol of miRNeasy FFPE Kit and stored at −80 °C until microdissection with the use of a laser microdissection system (Leica Microsystems, Japan, Tokyo) and the RNA extraction.

2.4. RNA extraction

Prior to the extraction of total RNA from the sera, 0.2 fmol of synthetic miR-3 from *D. melanogaster* (dme-miR-3), which is posted on rat microarrays from Agilent Technologies as a negative control, was spiked in each sample as an internal standard. Total RNA was isolated from serum in accordance with the protocol for PARIS™ Kit (Applied Biosystems, Tokyo, Japan) with minor modification such that samples were extracted twice with an equal volume of acid–phenol chloroform because of abundant proteins. In terms of the extraction from the microdissected liver tissues, the procedure was conducted in accordance with miRNeasy FFPE Kit (QIAGEN, Tokyo, Japan). As a result of the RNA extraction from the sera, samples having very low extraction efficiency of the total RNA or insufficient quantity of total RNA were excluded from microarray data, and applied to qRT-PCR.

2.5. miRNA microarray and data analysis

MiRNA microarray analysis was conducted on each total RNA samples using a Rat miRNA microarray kit (Agilent Technologies, Santa Clara, CA). The procedure was basically conducted following the manufacturer's protocol (Version 2.0) and a Feature Extraction (Version 10.5) was used to generate a quantitative signal value and a qualitative detection call for each probe on the microarray (Agilent Technologies). The 75th percentile normalization was used for per sample normalization. Furthermore, for per miRNA normalization, four miRNAs (miR-16, 25, 146a, 221) showing that the CV values calculated from their signal values were rather small in two independent sample sets, i.e., the samples from the animals sacrificed at Week 26 or 36 was also conducted. We analyzed miRNA normalized expression data with Welch's *t*-test to obtain the miRNAs, which were significantly altered in comparison with the expressions in control samples. The obtained miRNAs were applied to a supervised hierarchical clustering analysis with GeneSpring GX 7.3.1 (Agilent Technologies). Pearson correlation and standard correlation were also used for the similarity measurement of genes and samples, respectively in the analysis.

2.6. Real-time quantitative RT-PCR of mature miRNAs

Real-time quantitative RT-PCR (qRT-PCR) of mature miRNAs was conducted in accordance with the protocol of TaqMan® MicroRNA Assays using 7500 Fast Real-Time PCR Systems in Applied Biosystems. All reactions were run in duplicate. After reaction, the C_T data were determined using default threshold settings, and after the correction of the C_T data with the extraction efficiency of the total RNA mentioned below, the mean C_T was determined from the duplicate PCRs. The ratio of circulating miRNA of rat with the lesion to normal circulating miRNA was calculated by using the equation $2^{-\Delta G}$, in which $\Delta G = C_{T\,\text{lesion}} - C_{T\,\text{normal}}$. The extraction efficiency of total RNA was obtained from the concentration of the spiked miRNA, i.e., dme-miR-3 in total RNAs isolated from the serum, divided by the concentration spiked, 0.2 fmol. All primers used were purchased from Applied Biosystems. For analysis of the all data, Bartlett's test in ARM EXSUS ver. 7.6 (Arm systex Co. Ltd., Osaka, Japan) was applied to test the homogeneity of variance between the groups. When the groups were accepted to be homogeneous, Dunnett's multiple comparison procedure in the same package was used for comparison of groups of data. When the groups of data were heterogeneous in Bartlett's test, Steel's test in the same package was used. Statistical significance was evaluated at $P < 0.05$ and 0.01. Furthermore, Jonckheere's test was also applied to analyze the tendency between the levels of selected circulating miRNAs and the progress of the hepatic lesions from moderate or severe FHA to HCC.

3. Results

3.1. Histopathological examination

The results of histopathological examination of the livers from the rats sacrificed at Week 26 are shown in Table 1. Mild FHA, which represents one or more FHA sometimes observable within a hepatic lobule, was observed in 5 rats treated with DEN alone. Moderate FHA, one or more FHA observable frequently within a hepatic lobule, was observed in 2 rats treated with DEN + PB and 3 rats treated with DEN + clofibrate. Severe FHA, representing one or more large

Table 1
Histopathological findings in liver.

Histopathological findings[a]	Breeding term	26 weeks					36 weeks				
		1[b]	2[b]	3[b]	4[b]	5[b]	1[b]	2[b]	3[b]	4[b]	5[b]
Number of animals examined		5	5	5	5	5	5	5	5	5	5
No remarkable findings		5	0	0	0	0	4	0	0	0	0
Hepatocellular hypertrophy, centrilobular	+	0	0	4	3	0	0	0	0	2	0
	2+	0	0	1	2	0	0	0	5	3	0
Eosinophilic change in cytoplasm, hepatocyte	+	0	0	0	0	5	0	0	0	0	5
Foci of hepatocellular alteration	+–	0	0	0	0	0	1	0	0	0	0
	+	0	5	0	0	0	0	0	0	0	0
	2+	0	0	2	0	3	0	1	0	0	0
	3+	0	0	3	5	2	0	4	5	5	5
Hepatocellular adenoma		0	0	1	0	1	0	2	0	0	3
Hepatocelllular carcinoma		0	0	0	5	0	0	1	5	5	2

[a] Grading: +–, slight; +, mild; 2+, moderate; 3+, severe.
[b] Group 1, control (no treatment); Group 2, DEN; Group 3, DEN + PB; Group 4, DEN + DDT; Group 5, DEN + clofibrate.