Technical Notes

Detection of MicroRNA Profile with Bead–based Microarray in Hepatocellular Carcinoma Cell Line HepG2 *

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ABSTRACT **Objective:** To investigate the application of bead-based Luminex® xMAP™ technology and to detect the variation of microRNA (miRNA) expression profile in hepatocellular carcinoma (HCC) HepG2 cells compared to normal hepatocytes. **Methods:** Four hundred and twenty-six miRNAs were labeled and determined using bead-based flow-cytometric microarray platform with total RNA extracted from HepG2 and normal hepatocytes. Fluorescence intensity values were measured with a Luminex 100IS machine. Four miRNAs were selected to be conformed by real-time quantitative RT-PCR (qRT-PCR). **Results:** Bead-based Luminex® xMAP™ technology succeeded in exploring the miRNA profiles with HepG2 and normal hepatocytes. Twenty-eight miRNAs were detected to be downregulated and 45 upregulated, including a part of miRNAs being reported previously. Detections of 4 miRNAs with real-time qRT-PCR were consistent with bead-based miRNA microarray.

Conclusion: Measurement of individual miRNA by the bead-based method is feasible, high speed, sensitive and economic. This technology can be proposed as an alternative method to qRT-PCR for validating miRNA expression data obtained with high-throughput technologies. The variation of the miRNA profiles between HCC and normal hepatic cells indicated that a certain amount of miRNAs may be involved in the hepatocarcinogenesis.

Key Words: MicroRNAs; HCC; Bead-based, Microarray; Real-time quantitative RT-PCR

Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive human malignancies, common in Asia, Africa, and the areas with endemic infections of hepatitis-B or -C viruses (HBV or HCV) [1-2] . It has the third highest mortality rate among cancers worldwide and the second highest cancer mortality rate in China since the 1990s. Globally, the 5-year survival rate of HCC is <5% and about 600, 000 HCC patients die each year[3]. The high mortality associated with this disease is mainly attributed to the failure to diagnose HCC patients at the early stage and the lack of effective therapies for patients at advanced stage. Although surgery remains the most effective treatment for HCC, the majority of patients are inoperable at presentation because of late diagnosis [4-5]. The consequent improvement in long-term survival of post-surgery patients is only modest because of a high recurrence rate of intra-hepatic metastases through invasion of the portal vein or spread to other parts of the liver. Understanding the relationships between phenotypic and molecular changes in HCC is of great importance to develop new diagnosis and treatment of HCC and improve the prognosis of diagnosed patients [2, 5-8].

Micro-ribonucleic acids (MicroRNAs, miRNAs) are an abundant class of 17–25 nucleotides small non-coding RNAs. They regulate protein-coding genes expression at the post-transcriptional level through binding to the 3'untranslational region (3' UTR) of target mRNAs. Since the initial observation, about 1000 miRNA sequences have been identified in mammals, but the biological functions of a large part remain illusive.

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Growing evidence have suggested that miRNAs have important roles in the regulation of diverse biological processes, and their deregulation or dysfunction participates in cancer development and clinical outcomes of cancer patients [9]. However, deregulated miRNAs and their roles in tumorigenesis remain still largely unknown. To date, miRNAs have been suggested to have important roles in HCC development [10], and some of them have been identified to correlate with prognosis or accepted to be potential therapeutic targets [11]. Unfortunately, elucidating miRNA deregulation or dysfunction in HCC development is still an ongoing process [12].

As conventional protein-coding genes, it is of interest to profile the expression patterns of microRNAs during the development of a disease. To profile gene expression, microarrays, consisting of arrayed series of thousands of microscopic spots of DNA oligonucleotides, have been the standard technology. Microarray platforms also exist for profiling microRNA expression [13]. However, at present, no standard methodology exists for hybridization-based profiling of miRNAs and, as a consequence, comparison of expression data from different experiments can be difficult. To solve these problems it will be necessary to develop quality procedures for miRNA microarrays. LuminexR xMAP™ system is a multiplexed microsphere-based suspension array platform capable of performing and reporting up to 100 different analyses in a single reaction vessel [14]. In particular, oligonucleotide-capturing probes complementary to miRNAs of interest are coupled to carboxilated 5-micron polystyrene beads impregnated with variable mixtures of two fluorescent dyes, each representing a single miRNA. Using this technique Lu et al. [15] were able to differentiate tumors that were instead inaccurately classified by miRNA profiles. Recently, a study performed with Luminex miRNA platform, identified new markers of human breast cancer subtype [16]. When compared to glass-slide microarrays, the bead-based miRNA arrays showed many advantages such as easy to use, low cost, superior statistical performance, faster hybridization kinetics (solution hybridization) and higher flexibility in array preparation. Furthermore, miRNA expression obtained from high throughput arrays has to be validated with alternative technologies. Real-time quantitative RT-PCR (qRT-PCR) has become the golden standard of miRNA quantification because it offers the highest sensitivity from small amounts of starting material and it is able to detect as little as 1-nt difference between miRNAs. So in the present study, 426 miRNAs were determined using bead-based flow-cytometric microarray platform with total RNA extracted from HepG2 and compared to those in normal hepatocytes. After that, 4 miRNAs were chosen to be detected for conformation by real-time qRT-PCR.

MATERIALS AND METHODS

Samples

The human hepatoma-derived cell lines HepG2 was purchased from the American Type Culture Collection *(ATCC, Rockville,MD, USA)* and cultured in Dulbecco's modified essential medium *(*DMEM, *Invitrogen Corp., Grand Island, NY, USA)*, supplemented with 10% heat-inactivated fetal bovine serum *(Invitrogen Corp., Grand Island, NY, USA)*, 2mM glutamine, gentamicin at 37℃ in a humidified incubator with 5% CO_2. Primary human hepatocytes were obtained from a male donor with normal liver tissue.

RNA extraction

RNA isolation was performed on the ABI PRISM® 6100 prep station using the AbsoluteRNA Solution *(Applied Biosystems, Lennik, Belgium)* to remove contaminating DNA and PCR inhibitory substances. RNA concentrations were determined using a ND-1000 NanoDrop *(Thermo Fisher Scientific, Wilmington, Delaware USA)*, and used for normalization of the input RNA in the real-time qRT-PCR.

MicroRNA labeling

MicroRNA labeling was performed as described previously [15-17]. In brief, miRNAs were extracted from 5 μg of sample total RNA using denaturing PAGE, samples were spiked with three synthetic pre-labeling control RNAs (5'-pCAGUCAGUCAGUCAGUCAGUCAG-3', 5'-pGACCUCCAUGUAAACGUACAA-3', 5'-pUUGCAGAUAACUGGUACAAG-3'; *Dharmacon, Lafayette,CO, USA*) to control for target preparation efficiency, at 3 fmoles/sample. After purification of 18-26 bp RNAs, adaptors were ligated at the 3' and 5' ends using T4 RNA ligase (*Fermentas, Burlington, OT, CA*), a RNA-DNA hybrid 5'-pUUUaaccgcgaattccagt-idT-3' (*Dharmacon; X = RNA, x =DNA, p = phosphate, idT = inverted [3'-3' bond] deoxythymidine*) was ligated to the 3' end and 5'-acggaattcctcactAAA-3' (*Dharmacon*) was ligated to the 5' end using T4 RNA ligase. These bi-ligated products underwent reverse transcription using an adaptor specific primer (M37, 5'-pTACTGGAATTCGCGGTTA-3') and then amplified and labeled using PCR (M37 and M33, 5'-biotin-CAACGGAATTCCTCACTAAA-3'). Amplification was performed on an Eppendorf thermal cycler at 95°C for 30 s, 50°C for 30 s and 72°C for 40 s for 18 cycles. PCR products were

precipitated without glycogen and redissolved in 66 µl 1 × TE buffer containing 1 µl of three biotinylated post labeling controls (100 fmols each).

Bead coupling and hybridization

Oligonucleotide probes were coupled to color-coded polystyrene beads, allowing the simultaneous detection of about 90 different target oligonucleotides. To obtain expression profiles for 426 miRNAs, 8 distinct sets of bead-coupled miRNA probes were created. Each sample was hybridized to the 8 bead sets to generate a complete miRNA profile. Oligos were 5'-amino modified with a 6-carbon linker and conjugated to carboxylated xMAP beads *(Luminex, Austin, TX, USA)* in 96-well formats following the standard manufacturer's protocol. To generate bead set pools, 3 µl of each oligobead conjugate was mixed into 1 ml 1.5× TMAC buffer (4.5 M tetramethylammonium chloride, 0.15% sarkosyl, 75 mM Tris-HCl pH 8.0, 6 mM EDTA). Samples were hybridized in a 96-well format with two water-only blanks and at least three bead blanks containing water instead of the labeled sample for use as a background control. Replicate probes and technical replicate samples across bead sets and sample plates were included, respectively, to aid quality control and data preprocessing. Hybridization was carried out overnight at 50°C with 33 µl of the bead pool and 15 µl of labeled sample. Unbound sample was removed from beads by washing with 1× TE and resuspending in 1× TMAC buffer. SAPE, streptavidin-phycoerythrin, premium grade *(Invitrogen)* was added to the beads (1:100 dilution) and incubated for 10 minutes at 50°C to bind to biotin moieties on the cDNA. Samples were processed on a Luminex 100 machine and median fluorescence intensity values were acquired using the StarStation software *(ACS, Sheffield, UK)*.

Computational Preprocessing

Median fluorescence intensity values smaller than a threshold of 1 were set equal to 1, and all values were transformed by taking logs. To reduce noise due to absent probes, each probe was required to exceed a log2 median fluorescence intensity value of 6 in at least one sample. Systematic probe effects were median-corrected. Triplicate probes were summarized by their mean profile and samples were centered to have zero median. Technical triplicate samples were summarized by their mean profile. The relative expressed miRNAs change fold of HepG2 was calculated by comparing to the miRNAs expression of normal hepatocytes.

Real–time quantitative RT–PCR

Expressions of 4 selected miRNAs, including hsa-miR-126, hsa-miR-122a, hsa-miR-125a, and hsa-miR-20a, were validated with TaqMan MicroRNA Assays according to the manufacturer's instructions *(Applied Biosystems, Foster City, CA, USA)* and normalized to the average expression of U6 small nuclear RNA (RNU6), hsa-miR-191, and hsa-miR-103 *(Applied Biosystems)*. Real-time qRT-PCR reactions and data acquisition were performed on the ABI Prism 7900HT sequence detection system *(Applied Biosystems)*. Quantitative values were obtained from the threshold PCR cycle number (Cp) at which point the increase in signal associated with an exponential growth for the PCR product was detected. The target miRNA abundance in each sample was normalized to its reference level as $\Delta Cp = Cp_{\text{target miRNA}} - Cp_{\text{reference}}$ and $\Delta\Delta Cp = \Delta Cp_{\text{hepatocytes}} - \Delta Cp_{\text{HepG2}}$. All experiments were performed in triplicate.

RESULTS

Profiling

The pattern of miRNA expression in HepG2 cells was markedly different from the normal hepatocytes. After normalization, the variation of the expression profile on 73 miRNAs was determined between HepG2 and normal hepatocytes, including 28 down-regulations and 45 up-regulations. Twenty dowregulated miRNAs with the change fold less than 0.5 were shown in Figure 1A and 35 upregulated miRNAs with the change fold higher than 8 were summarized in Figure 1B.

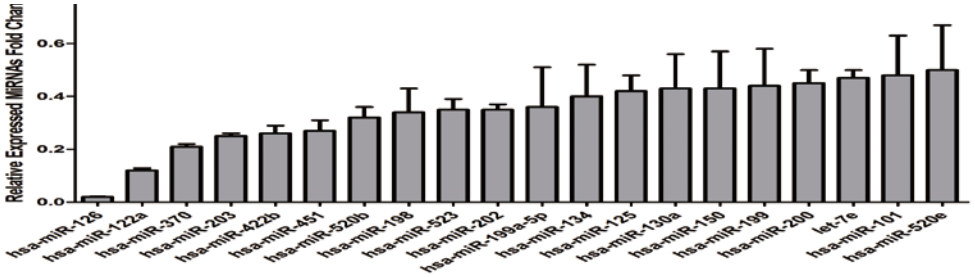

Figure 1A

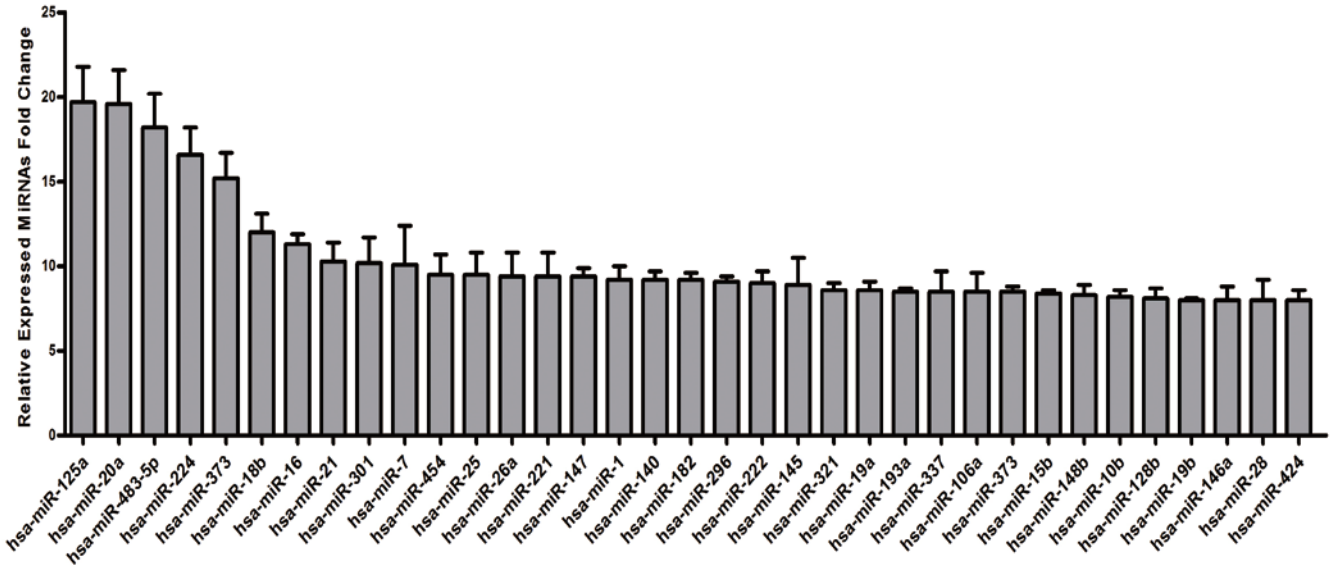

Figure 1B

Figure 1 Dysregulation of the MicroRNA profile using bead-based Luminex® xMAP™ technology in HepG2

Real-time qRT-PCR

Hsa-miR-126, hsa-miR-122a, hsa-miR-125a, and hsa-miR-20a were selected for further study because they were the most differently expressed miRNA in the present profiling study in HepG2. The expression level of hsa-miR-126 and hsa-miR-122a was only or lower than 10% compared to the normal hepatocytes. Meanwhile, hsa-miR-125a, and hsa-miR-20a showed nearly 20 fold increases. To determine the validity of the miRNA microarray, we verified hsa-miR-126, hsa-miR-122a, hsa-miR-125a, and hsa-miR-20a expression in HepG2 cells and hepatocytes by real-time qRT-PCR (Figure 2). The result was in agreement with the expression pattern with the bead-based miRNA microarray. △△Cp of hsa-miR-126, hsa-miR-122a, hsa-miR-125a, and hsa-miR-20a was (-8.1±0.9), (-6.8±0.73), (6.2±0.9), and (5.9±0.45), respectively.

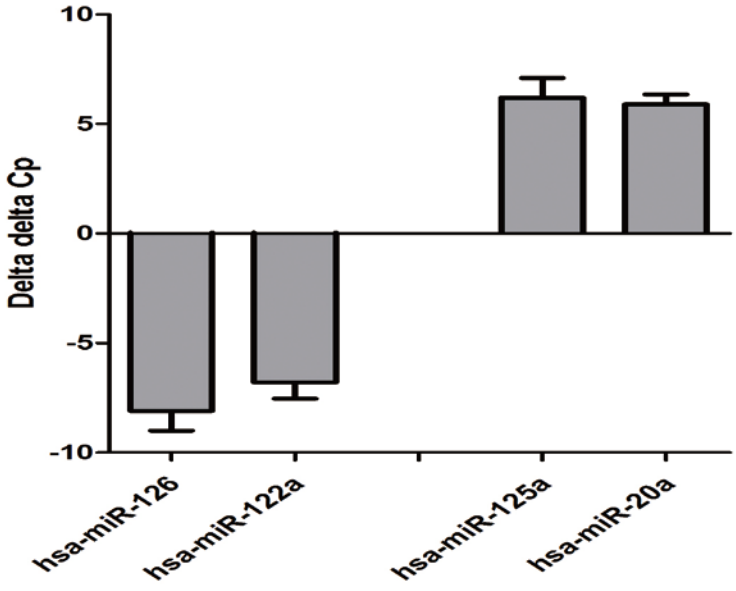

Figure2 Dysregulation of the MicroRNAs with quantitative real-time qRT-PCR in HepG2

DISCUSSION

There are currently different microarrays available for miRNA profiling and glass-slide microarrays have been widely used. However, cross-hybridization of related miRNAs has been problematic. In the present study with the bead-based Luminex® xMAP™ miRNA microarray technology, oligonucleotide-capture probes complementary to miRNAs of interest were coupled to carboxylated 5-micron polystyrene beads impregnated with variable mixtures of two fluorescent dyes, each representing a single miRNA. Following adaptor ligations which use both the 5'-phosphate and the 3'-hydroxyl groups of miRNAs, reverse transcribed miRNAs were amplified by PCR with a common biotinylated primer, then hybridized to the capture beads, and followed to be stained with streptavidin-phycoerythrin. The beads were then analyzed using a flow cytometer capable of measuring bead color (denoting miRNA identity) and phycoerythrin intensity (denoting miRNA abundance) . Bead-based hybridization has the advantage that it might more closely approximate hybridization in solution. Lu et al [15] performed a spiking experiment involving 11 related sequences showed increased specificity of bead-based detection compared with microarray-based detection, even for single base-pair mismatches. In addition, bead-based detection paralleled the data from northern blotting [15]. In the present study, 4 candidates from the miRNA profile were also conformed by real-time qRT-PCR, which demonstrates that bead-based miRNA microarray detection is feasible, and has the attractive properties of improved accuracy, high speed and low cost. The bead-based detection platform also provides flexibility, in that additional miRNA capture beads can be added to the mixture, allowing detection of newly discovered miRNAs. Moreover, the bead-based miRNA detection method has the attractive property of being not only accurate and specific, but also easy to implement in a routine clinical setting. In addition, unlike mRNAs, miRNAs remain largely intact in routinely collected, formalin-fixed, paraffin-embedded clinical tissues. More work is required to establish the clinical utility of miRNA expression in cancer diagnosis and prognosis.

Li et al. [18] recently compared miRNA expression profiles between HepG2 and LO2 immortalized hepatic epithelium cell line with another miRNA microarray technique. The expression profile of 143 miRNAs was found to be dyeregulated, more than the 73 miRNAs detected in the present study. It could be partly explained by the difference between the microarrays and the materials. As what was expected, there was some overlapping between the two individual studies. Hsa-miR-18b, hsa-miR-301, hsa-miR-193a, hsa-miR-224, hsa-miR-148b, hsa-miR-19a, hsa-miR-28 and hsa-miR-424 were found to be increased expressed in both studies. Meanwhile, hsa-miR-122a, hsa-miR-520e, hsa-miR-134, hsa-miR-451, hsa-miR-198, hsa-miR-520b, hsa-miR-523, hsa-miR-202 and hsa-miR-200 were detected to be decreased expressed in both studies, but with different degrees. This conformation indicated that the aberrant expression of different miRNAs may play an important role in the tumorogenisis of HCC.

Among the miRNAs of the present profile, some have previously been detected in the clinical HCC samples or studied with *in vitro* functional experiments. Hsa-miR-122, a hepatospecific miRNA, is frequently downregulated in Hep3B and HepG2 cell lines, and regulates endogenous apoptosis via the Bcl-w gene [19]. Gramantieri et al. found that cyclin G1 was one of the hsa-miR-122a target genes[20]. Decreased expression of hsa-miR-101 was detected all 6 hepatoma cell lines examined and in as high as 94.1% of HCC tissues, compared with their non-tumor counterparts by Su et al.[21]. Furthermore, ectopic expression of hsa-miR-101 dramatically suppressed the ability of hepatoma cells to form colonies *in vitro* and to develop tumors in nude mice [21]. But Li et al. [18] reported that hsa-miR-101 was increased expressed in HepG2, which was opposite to other report[21] and our present study. Besides the miRNAs mentioned above, hsa-miR-125, hsa-miR-126, hsa-miR-130a, hsa-miR-150, hsa-miR-199a-5p, hsa-miR-200, and let-7 family members were also discovered to be downregulated in HCC[10 ,22-25]. Meanwhile, a number of miRNAs were determined to be upregulated in HCC cells or tissues. Murakami et al.[23] found that hsa-miR-224 was upregulated in HCC tissues compared with adjacent non-tumor liver tissues and Li et al. [18] discovered that cell proliferation, migration and invasion were altered after changing the expression of hsa-miR-224 in HepG2. Other studies reported that hsa-miR-18, hsa-miR-21, hsa-miR-146a, hsa-miR-221, hsa-miR-222, hsa-miR-301 and hsa-miR-373 were detected to be upregulated[26-28]. No previous studies investigated the expression of hsa-miR-370, hsa-miR-451, hsa-miR-125a, hsa-miR-20a, hsa-miR-483-5p in HCC, all of which were detected to be dysregulated in the HCC cell line HepG2 in the present study. The future study of these miRNAs may provide additional insight into the role of miRNAs in HCC.

Taken together, detection of miRNAs by the bead-based miRNA microarray method is feasible, high speed, sensitive and low cost. This technology can be proposed as an alternative method to qRT-PCR for validating miRNAs expression data obtained with high-throughput technologies. MiRNA expression patterns may potentially become useful markers for HCC diagnosis, classification and prognostic risk stratification.

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