A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA)

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ABSTRACT

MicroRNAs represent a class of short (~22 nt), noncoding regulatory RNAs involved in development, differentiation, and metabolism. We describe a novel microarray platform for genome-wide profiling of mature miRNAs (miChip) using locked nucleic acid (LNA)-modified capture probes. The biophysical properties of LNA were exploited to design probe sets for uniform, high-affinity hybridizations yielding highly accurate signals able to discriminate between single nucleotide differences and, hence, between closely related miRNA family members. The superior detection sensitivity eliminates the need for RNA size selection and/or amplification. MiChip will greatly simplify miRNA expression profiling of biological and clinical samples.

Keywords: microRNAs; microarrays; LNA; RNA translation; mouse

INTRODUCTION

MicroRNAs (miRNAs) constitute a class of recently discovered short regulatory RNAs that control gene expression post-transcriptionally in, e.g., development, differentiation, and metabolism (Krichevsky et al. 2003; Abbott et al. 2005; Chen and Lodish 2005; de Moor et al. 2005; Harfe 2005; Leaman et al. 2005). miRNAs are initially transcribed as long precursor RNA molecules (pri-miRNAs) and successively processed by Drosha and Dicer complexes into their mature forms of ~22 nt.

Cloning efforts and bioinformatic predictions suggest that miRNAs may regulate up to 20%–25% of mammalian genes (Lewis et al. 2005). miRNAs control RNA expression at the level of turnover and/or translation via base-pairing, usually to their 3′-untranslated regions (Lee et al. 1993; de Moor et al. 2005). Both the qualitative and the quantitative expressions of miRNAs, therefore, are expected to exert a profound regulatory influence on the transcriptome of a given cell or tissue (Doench and Sharp 2004; de Moor et al. 2005). The accurate profiling of miRNA expression thus represents an important tool to investigate physiological and pathophysiological states.

Different methodologies have been used to profile miRNA expression, including Northern blotting with radiolabeled probes (Sempere et al. 2004; Valoczi et al. 2004), oligonucleotide macroarrays (Krichevsky et al. 2003; Sioud and Rosok 2004), quantitative PCR-based amplification of precursor or mature miRNAs (Schmittgen et al. 2004; Jiang et al. 2005; Shi and Chiang 2005), bead-based profiling methods (Barad et al. 2004; Lu et al. 2005), and DNA microarrays spotted onto glass surfaces (Babak et al. 2004; Liu et al. 2004; Miska et al. 2004; Nelson et al. 2004; Thomson et al. 2004; Baskerville and Bartel 2005). By contrast to mRNA profiling technologies, miRNA profiling must address the short nature (~22 nt) of miRNAs and should be able to distinguish between miRNAs that differ by as little as a single nucleotide.

Microarray-based miRNA profiling assays constitute an efficient methodology to screen in a parallel fashion for the expression of a large number of miRNAs through extensive sample collections. While reliable expression profiles can be obtained on most of the experimental platforms described so far, their use can be limited by the large amount of starting material needed (Miska et al. 2004; Thomson et al. 2004), work-intensive miRNA enrichment procedures (Miska

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et al. 2004; Baskerville and Bartel 2005), and/or amplification and labeling protocols that can introduce quantitative bias and, hence, experimental error (Barad et al. 2004; Liu et al. 2004; Miska et al. 2004; Baskerville and Bartel 2005). In addition, discrimination between single nucleotide differences is not always possible (Miska et al. 2004; Baskerville and Bartel 2005).

We describe a novel microarray platform (miChip) that accurately and sensitively monitors the expression of miRNAs without prior need for RNA size fractionation and/or amplification and that can discriminate among closely related miRNA family members. The described technology is based on the use of locked nucleic acid (LNA)-modified oligonucleotides that were previously recognized for their superior performance in Northern blotting and miRNA-specific in situ hybridization assays (Valoczi et al. 2004; Thomsen et al. 2005; Nelson et al. 2006). LNA is a synthetic RNA/DNA analog characterized by increased thermostability of nucleic acid duplexes when LNA monomers are introduced into oligonucleotides (Braasch and Corey 2001).

We hypothesized that LNA-modified capture probes (1) may result in a more sensitive detection of miRNAs in comparison to unmodified DNA-based capture probes and (2) can be designed such that an uniform melting temperature (T_m) can be applied to a genome-wide set of miRNAs by adjusting the LNA content and the length of the capture probes (Baskerville and Bartel 2005). T_m normalization of capture probes permits to establish normalized hybridization conditions suitable for all miRNAs, which cover a range of T_ms between 45°C and 74°C. Hybridization conditions optimized for high stringency binding not only accurately detect the expression of miRNAs but also increase the hybridization specificity for related miRNA family members that often differ by as little as a single nucleotide (Griffiths-Jones 2004).

RESULTS

To establish a microarray platform that accurately and sensitively measures the expression levels of miRNAs without need for RNA size fractionation and/or RNA amplification, we optimized RNA preparation protocols, coating chemistries for glass surfaces, chemical modifications of capture probes, as well as labeling and hybridization protocols.

Locked nucleic acid (LNA)-modified DNA oligonucleotides display superior sensitivity

More than 300 human and mouse miRNAs (miRBase release 7.1) have been annotated in the miRNA registry at the present (Griffiths-Jones 2004). Their calculated T_ms toward the complementary DNA strand vary between 45°C and 74°C, prohibiting uniform, equally effective hybridization conditions for the entire set of miRNAs based on capture probes of standard composition. At a medium hybridization temperature (e.g., 65°C), capture probes with lower T_m values will yield lower signals, while capture probes with higher T_m values will display impaired nucleotide discrimination and, hence, lower specificity.

To overcome these limitations, we spotted a test set of capture probes on coated glass surfaces, which consist of either unmodified DNA oligonucleotides or of a defined combination of unmodified and LNA-modified nucleotide analogs (Tolstrup et al. 2003). The LNA modification raises the thermostability of nucleic acid duplex by up to 4°C per nucleotide when the complementary strand is DNA, and up to 8°C when the complementary strand is RNA (Nielsen et al. 2003). In a first approach, we compared unmodified and LNA-modified capture probe test sets of identical sequences (see Supplemental Table 1). The capture probes were spotted onto N-hydroxysuccinamide (NHS)-coated (CodeLink, GE Healthcare) slides, which proved to possess superior properties regarding the binding of short (20 mer) oligodeoxynucleotides in preliminary experiments (see Supplemental Fig. 1A,B; Supplemental Table 4).

Arrays were hybridized to total murine liver RNA directly labeled at the 3′-end by ligation of a short, Cy-dye modified linker sequence (Fig. 1; see Materials and Methods). The hybridization conditions were separately optimized for the immobilized DNA and LNA-modified capture probes, respectively. The miRNA profile obtained from DNA-based capture probes is similar to the one generated by capture probes including LNA-modified nucleotides and consistent with previously published data (Fig. 1; Babak et al. 2004; Liu et al. 2004; Sempere et al. 2004; Thomson et al. 2004; Baskerville and Bartel 2005). However, LNA-modified capture probes yield a several-fold-increased hybridization signal in comparison to the unmodified DNA capture probes when the same amount of input RNA is used and identical labeling protocols are employed. The higher sensitivity of the LNA-modified capture probes is most obvious when lower amounts (2.5–5 μg) of total RNA are used. These results show that LNA-modified capture probes are more sensitive than DNA capture probes, offering superior performance for miRNA detection.

T_m-normalized capture probes facilitate mismatch discrimination

To benefit from the biophysical properties offered by the LNA-modification, capture probes with a uniformly normalized T_m of 72°C were designed. Probe design included the adjustment of both the length and the LNA content of individual capture probes (see Supplemental Table 2). A capture probe test set for these miRNAs was spotted onto NHS-coated glass slides and subsequently assessed for their accuracy in detecting miRNA expression in murine heart and liver samples. Figure 2 shows that these T_m-normalized LNA-modified capture probe sets generate tissue-specific

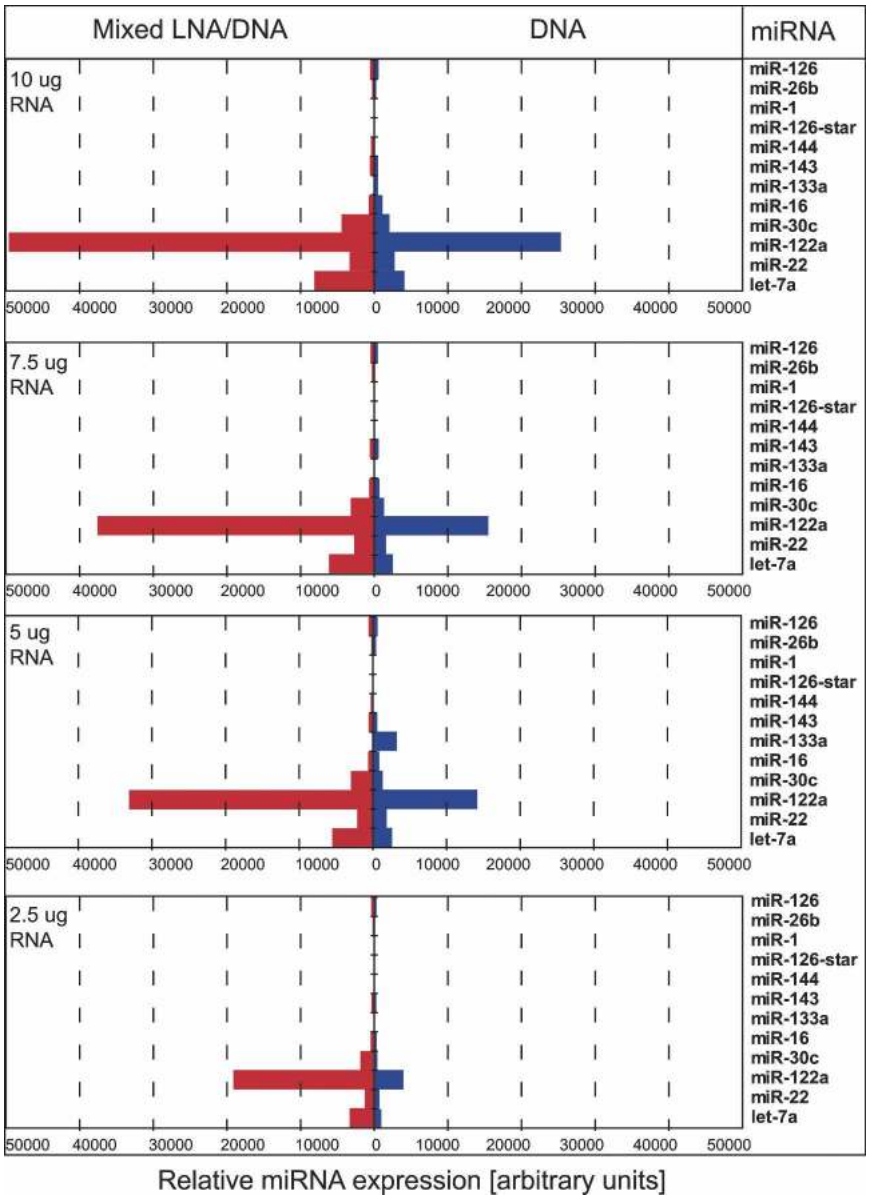

FIGURE 1. Mixed DNA/LNA capture probes display increased sensitivity for miRNA detection. miRNA expression was assessed in murine liver using a test set of LNA-modified (*left*) or unmodified DNA oligonucleotide capture probes (*right*). Decreasing amounts of total RNA were used as input material for miRNA analysis. Data are presented as median intensity (four replicas per miRNA capture probe; a representative experiment is shown).

miRNA profiles that reflect the known expression of tissue-specifically expressed miRNAs. For example, miR-122a expression is detected in liver, while miR-1 and miR-133a expression is not. In contrast, the heart expresses miR-1 and miR-133a but not miR-122a (Babak et al. 2004; Liu et al. 2004; Sempere et al. 2004; Thomson et al. 2004; Baskerville and Bartel 2005).

miRNA family members are often closely related and sometimes differ by as little as 1 nt in sequence. Because sequence-related miRNA family members may be involved in different physiological functions (Abbott et al. 2005; Leaman et al. 2005), it is important to be able to discriminate between such related miRNA family members. To assess whether T_m-normalized LNA-modified capture probes can distinguish between closely related sequences under our optimized hybridization conditions, we introduced single (1MM) and double mismatches (2MM) at the central position of the oligonucleotide (Barad et al. 2004; see Supplemental Table 2). miRNAs that yield high signal intensities after hybridization to LNA-modified capture probes with 100% complementarity (perfect match [PM]) in most cases (e.g., Let-7a, miR-30c, and miR-1) show a drastically reduced (often below background) hybridization signal when hybridized to the corresponding 1MM capture probe. In contrast, low-intensity hybridization signals from miRNAs that are not expressed in a given tissue (e.g., miR-122a in the heart and miR-1 in the liver, respectively) do not differ significantly between PM, 1MM, and 2MM capture probes, suggesting that they reflect background noise. Most importantly, two closely related miRNA family members that differ by a single nucleotide at position 9 (from the 5′-end) and a single nucleotide extension at the 3′-end (let-7a and let-7e) differ considerably in the signal intensities obtained in both liver and heart tissues (Fig. 2). These data demonstrate that the T_m-normalized LNA-modified capture probes hybridize their miRNA targets in a highly specific manner and that a single nucleotide mismatch suffices for significant destabilization of the heteroduplex. Thus, LNA-modified capture probes enable efficient discrimination between miRNA family members, at least when they differ in nucleotides close to the central position.

Confirmation of miChip data by Northern blotting

Northern blot analysis offers both quantitative and qualitative information about the expression of individual miRNAs (Sempere et al. 2004). We thus selected eight miRNAs from our test set that display either high (e.g., Let-7a and miR-122a) or low (e.g., miR-126) signal intensities, or where high (e.g., miR-122a and miR-1) or low (e.g., miR-22 and miR-30c) differential expression values between

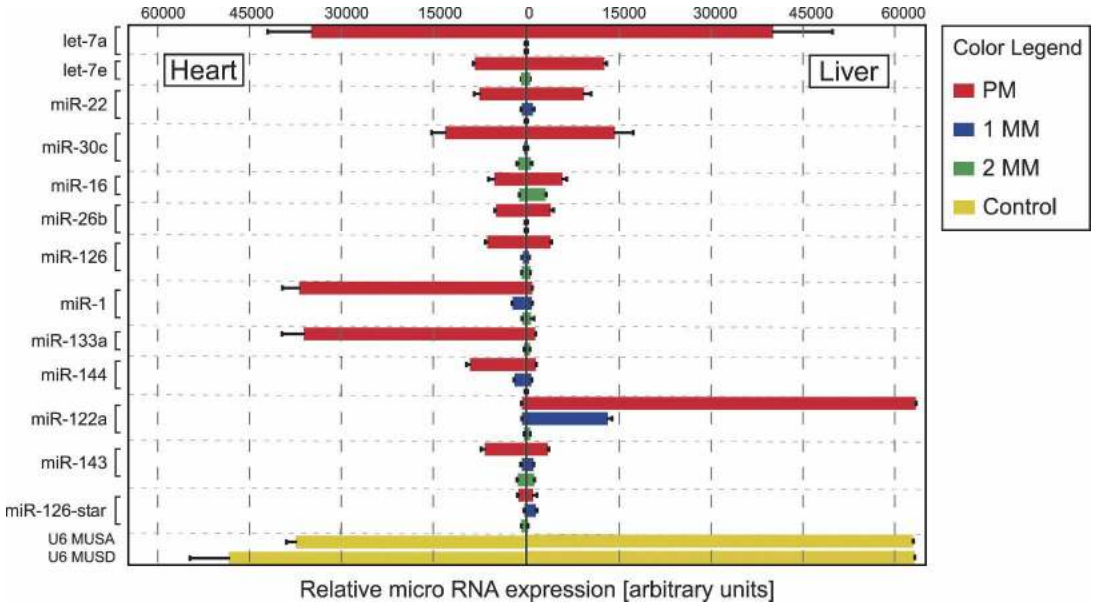

FIGURE 2. Accurate miRNA detection in murine heart and liver samples using T_m normalized LNA-modified capture probes. Detection of miRNA expression by PM (perfect match; 100% complementarity to the miRNA sequence), 1MM (single nucleotide mismatch at the central position of the nucleotide sequence of the mature miRNA), and 2MM (two nucleotide mismatches at the central position) LNA-modified captures probes. Data are presented as median intensity ± SD (four replicas per miRNA capture probe; a representative experiment is shown).

murine liver and heart were obtained by miChip analyses, and assessed their expression on Northern blots. The expression profiles obtained by Northern blotting fully supported the results of the miChip analysis (Fig. 3). Minor discrepancies (e.g., for miR-122a) may be caused by the higher specific activity of radioactively labeled probes that are used for the hybridization of Northern blots. Even the low differential expression observed between heart and liver for miR-22 and miR-30c is validated by Northern analysis. While most miRNAs monitored in Figure 3 are predominantly detected in their mature forms, the majority of the let-7e signal comes from a band that corresponds in size to the precursor miRNA (~65 nt). Interestingly, the let-7e hybridization signal measured on miChip correlates best with the one corresponding to the mature miRNA identified by Northern blotting. It is therefore likely that miChip analysis mainly detects mature miRNAs and discriminates somewhat against the respective precursor forms. We speculate that this discrimination may be caused by a more efficient ligation of the Cy-dye-labeled RNA oligonucleotides to the small mature miRNAs compared to the larger pre-miRNA molecules.

Genome-wide miRNA profiling using miChip

Based on our findings with the test set of LNA-modified capture probes, we next developed a genome-wide miChip microarray platform encompassing the 250 miRNAs contained in the miRBase, release 6.0 (Griffiths-Jones 2004), for mouse and human species (see Supplemental Table 3). All capture probes were normalized to a T_m of 72°C and spotted onto NHS-coated glass slides. To assess miChip accuracy, selected Murine tissues (duodenum, spleen, heart, and liver) for which the miRNA expression patterns are known were compared (Fig. 4). The data demonstrate that the miRNA profiles accurately reflect the known miRNA expression patterns in different tissues (Babak et al. 2004; Liu et al. 2004; Sempere et al. 2004; Thomson et al. 2004; Baskerville and Bartel 2005; see Supplemental Table 5).

DISCUSSION

miRNAs are small, noncoding RNAs that post-transcriptionally regulate gene expression (Krichevsky et al. 2003; Chen and Lodish 2005; de Moor et al. 2005; Harfe 2005). Their central biological roles suggest that their expression may provide valuable diagnostic and prognostic indicators for human diseases, including solid tumors and leukemias (Calin et al. 2004; Eckhardt et al. 2005; Eder and Scherr 2005; Iorio et al. 2005). Moreover, cellular miRNAs are also important for the replication of pathogenic viruses, and small RNAs are also encoded by the genomes of several viruses (Cai et al. 2005; Pfeffer et al. 2005). Additionally, it has been recently reported that human hepatitis virus C, after infection, needs a cellular miRNA (miR-122a) to facilitate its own replication, suggesting that miRNAs may represent novel targets for anti-viral intervention (Jopling et al. 2005).

Here we describe a novel microarray platform (miChip) that enables highly parallel profiling of miRNA gene expression in mammalian cells and tissues. We established time-efficient, easy-to-handle experimental protocols that also can be applied in clinical settings. The miRNA extraction

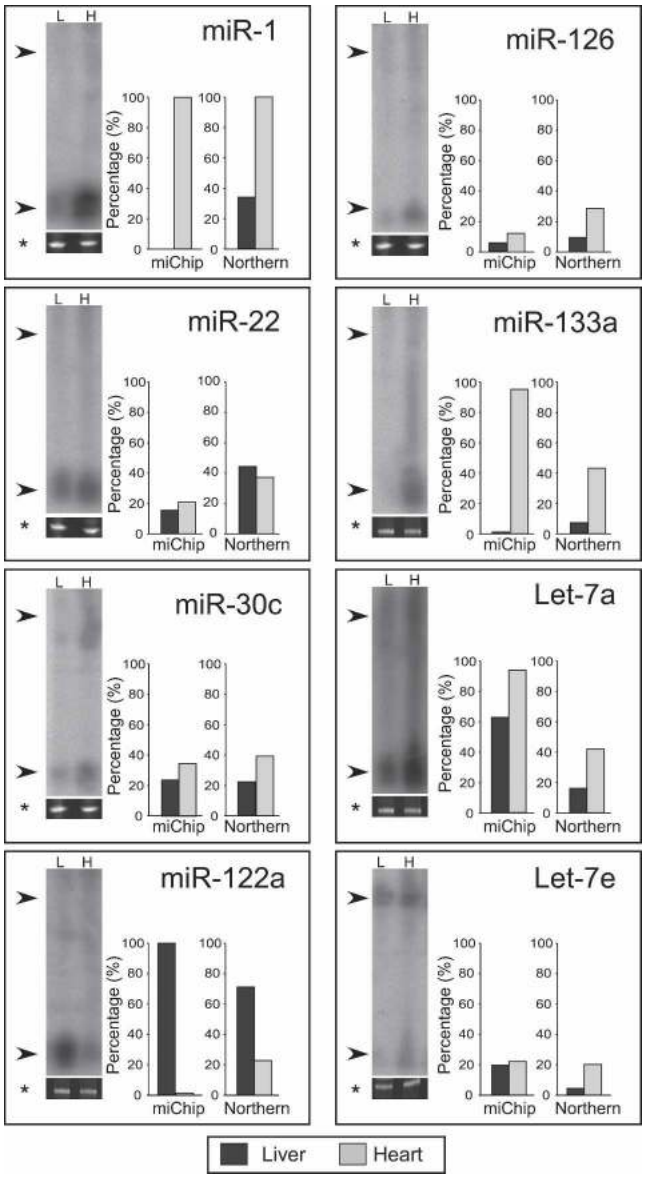

FIGURE 3. Confirmation of miChip data by Northern blotting. Eight miRNAs were selected for comparative analysis as described in the text. The *Y*-axis of the miChip graph refers to the background-corrected averaged mean fluorescence intensities of four replicas normalized for U6 RNA expression, while the *Y*-axis in the Northern blot graph refers to background-corrected intensities (as calculated by ImageJ). For both the miChip and the Northern blot data, the signal corresponding to the miRNA with the highest signal intensity (miR-1) was set to 100%, and signals for the additional miRNAs analyzed were calculated as a percentage thereof. Ethidium bromide stained 5S RNA (*) is shown as a loading control. Arrows indicate the position of the pre-miRNAs (*top* arrow, ~65 nt) and of the mature miRNAs (*bottom* arrow, ~22 nt).

and labeling protocols were optimized to allow the use of as little as 2.5 μg of total RNA as starting material without a need for miRNA enrichment and amplification. We consider these characteristics to be important advantages of this technology.

A major improvement of the sensitivity and accuracy of miChip was achieved by the use of mixed DNA/LNA-modified capture probes. The elevated thermal duplex stability (up to 8°C per LNA modified base) (Nielsen et al. 2003) enabled us to design mixed DNA/LNA capture probes with normalized melting temperatures by modifying LNA content and oligonucleotide length. The hybridization temperature of 55°C allowed the optimization of specific hybridization conditions (see Materials and Methods) suitable for the genome-wide miRNA set (based on miRNA registry release 6.0; Griffiths-Jones 2004).

MiChip analyses based on T_m-normalized, LNA-modified capture probes spotted onto NHS-coated glass slides circumvent many of the limitations associated with other microarray platforms for miRNA profiling that use exclusively unmodified DNA capture probes (Babak et al. 2004; Barad et al. 2004; Liu et al. 2004; Miska et al. 2004; Nelson et al. 2004; Thomson et al. 2004; Baskerville and Bartel 2005; Lu et al. 2005). These approaches often require larger amounts of starting material (Miska et al. 2004; Baskerville and Bartel 2005) or potentially error-prone amplification protocols as well as work-intensive miRNA enrichment procedures (Barad et al. 2004; Liu et al. 2004; Miska et al. 2004; Baskerville and Bartel 2005). The results obtained with the miChip microarray platform demonstrate that (1) the signal-to-noise ratio obtained with LNA-modified capture probes is substantially higher than with DNA-based capture probes (Fig. 1); (2) miRNA expression patterns are obtained with high sensitivity and accuracy; (3) the starting material can be reduced to as little as 2.5 μg of total RNA without compromising the quality of the results (Fig. 1); (4) excellent discrimination between miRNAs with single nucleotide differences can be achieved (Fig. 2), allowing efficient discrimination between closely related miRNA family members (Figs. 2, 3). Based on these data we expect that miChip analysis can greatly facilitate miRNA expression profiling of biological and clinical samples (Fig. 4).

MATERIALS AND METHODS

RNA isolation and Northern blot hybridization

Adult mice (C57Bl6/J) were sacrificed by neck dislocation, and organs were collected and stored in liquid nitrogen. Total RNA was prepared using Trizol (Invitrogen) following the manufacturer's protocol, where isopropanol was replaced by ethanol for RNA precipitation. RNA quality was ascertained using an Agilent 2100 bioanalyzer (Agilent technologies). For Northern blot analysis, 10 μg of total RNA were loaded onto 15% acrylamide, 6 M urea, and TBE gels alongside an appropriate DNA marker. Following electrophoresis, RNA was transferred to a nylon membrane (Nitran-N) using semidry transfer (semiphor; Pharmacia Biotech). Membranes were then hybridized overnight at 50°C in hybridization buffer (5× SSC, 20 mM Na_2HPO_4, 5% SDS and 1× Denhardt's), together with LNA oligonucleotides (miRCURY LNA Array detection probes; Exiqon) that were previously labeled

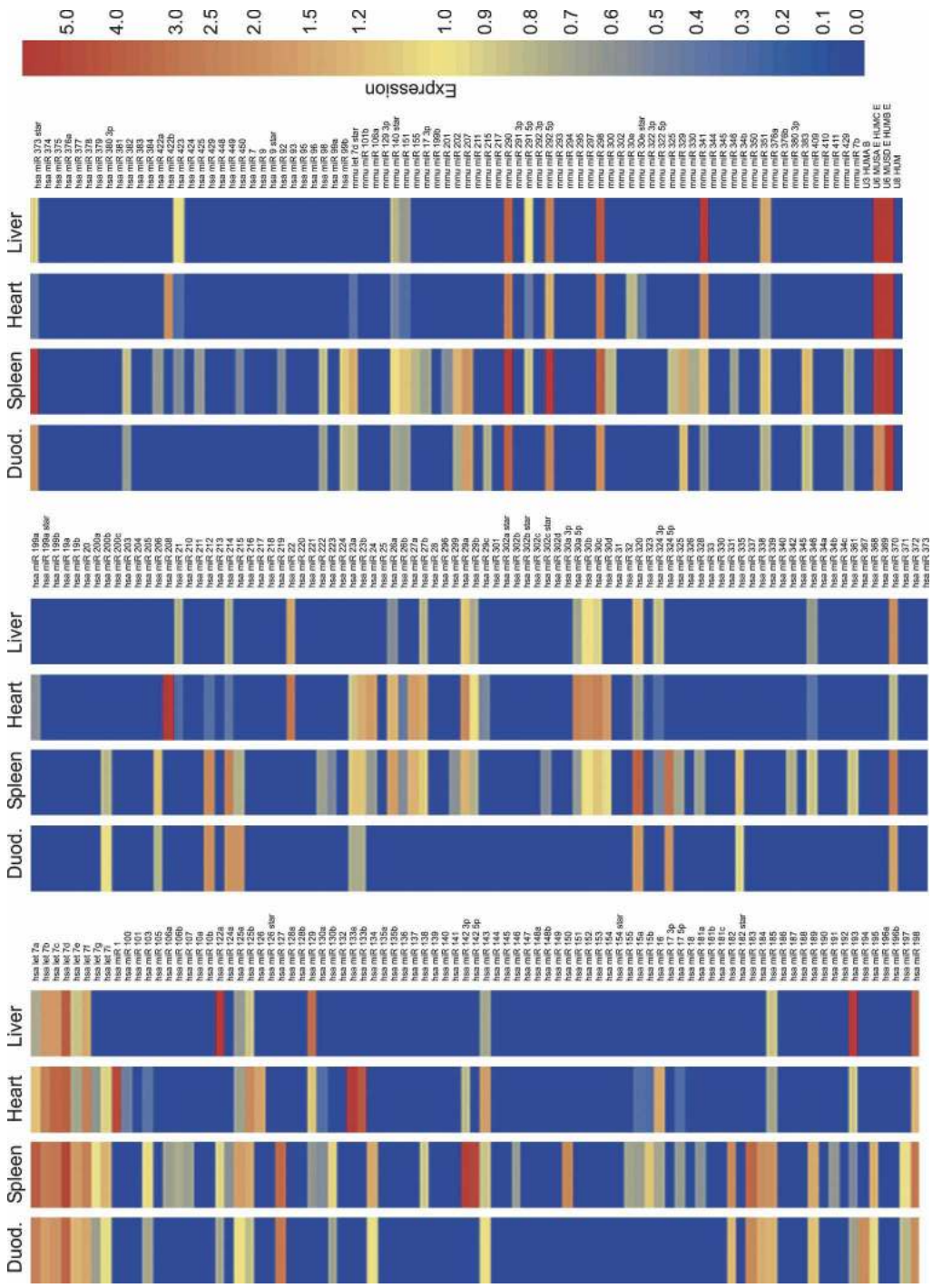

FIGURE 4. Genome-wide miRNA profiling using miChip. miRNA expression profiles were monitored across four mouse tissues (duodenum ["Duod."], spleen, heart, and liver). Data were organized according to the expression levels of individual miRNAs (condition tree). The key color bar indicates miRNA expression levels (dark red indicates high expression, while dark blue indicates no detectable expression; the Genespring data file is presented in Supplemental Table 5).

with polynucleotide kinase (NEB) and ^{32}P-ATP. Subsequently, the blot was washed twice at 50°C for 30 min (in 0.2× SSC) and exposed o/n on a phosphoimager (FLA-2000, Fujifilm).

Cy-dye labeling of miRNAs for microarray analysis

Total (2.5–10 μg) RNA was ligated to a RNA-linker (p-rUrUrUdA-Cy-dye [Miska et al. 2004], Dharmacon) labeled at the 3′-end with Cy3 or Cy5 using T4 RNA (NEB) ligase overnight at 37°C. Unbound nucleotides/RNA-linkers were removed by ethanol precipitation.

Microarray preparation and hybridization

DNA oligonucleotide (Sigma-Genosys) and LNA-modified oligonucleotide (Exiqon) capture probes containing a 5′-terminal C6-amino modified linker, were chosen for complementarity to the respective full-length mature miRNA sequences. A second set of LNA-modified oligonucleotides was designed to have uniform T_m of 72°C against their complementary targets, by adjustment of the LNA content and length of the capture probes (now available as miRCURY LNA Array probes; Exiqon [http://www.exiqon.com]) (see Supplemental Table 3). Capture probes were diluted to a final concentration of 20 μM in 150 mM phosphate-buffered saline (pH 8.5) and spotted on to N-hydroxysuccinamide glass surfaces (CodeLink; GE Healthcare). Slides were printed using either the OmniGrid 100 robot (GeneMachines) at 60%–65% humidity and 23°C–24°C, or the Lucidea Array Spotter (GE Healthcare) at 65% humidity and 21°C. Each spot measured ~120 μm in diameter with a center-to-center spacing between spots of 300 μm. Each probe was printed in quadruplicate using a re-dip after five slides. Spotted glass slides were subjected to post-processing as recommended by the manufacturer and stored desiccated until use.

Spotted microarray slides were processed using an automated hybridization station (Lucidea Slide Processor; GE Healthcare), which facilitates sample processing and hybridization standardization. To take the different hybridization kinetics of DNA and LNA-modified capture probes into account, we separately determined optimal hybridization conditions for LNA- and DNA-based arrays. Microarrays with immobilized LNA-modified capture probes were hybridized at 55°C using microarray hybridization solution (version 2; GE Healthcare) containing 30% formamide. Microarrays with immobilized DNA-oligonucleotides were hybridized at 42°C in hybridization solution (version 2; GE Healthcare) containing 10% formamide.

Image analysis and data processing

Slides were scanned using a Genepix 4000B laser scanner (Axon Instruments), where the photomultiplier (PMT) settings of the two channels were manually adjusted such that the intensity at A532 and A635 equals 1. Artifact-associated spots were eliminated both by software- and visual-guided flags. Image intensities were measured as a function of the median of foreground minus background. Negative values were normalized to 1. (If other normalization protocols were applied, they are described in the corresponding figure legends.) Microarray images were analyzed using the Genepix Pro 4.0 software (Axon Instruments). Excel and GeneSpring 7.2 (Silicon Genetics) were used for further data analysis. Medians of four background corrected replicas for each miRNA capture probe were uploaded into the GeneSpring microarray analysis software. Subsequently, each miChip was normalized to the 50th percentile of the positive controls (U6-MUSA and U6-MUSD) and to the median of the 50th percentile of total signal intensities on each array. Northern blot images were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

SUPPLEMENTAL DATA

Supplemental material can be found at http://www.embl-heidelberg.de/ExternalInfo/hentze/suppinfo.html and at http://www.klinikum.uni-heidelberg.de/index.php?id=6683/.

COMPETING INTERESTS

M.N. is an employee of Exiqon A/S. All other authors have no competing financial interests.

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