Appendix S1

SUPPLEMENTARY MATERIAL AND METHODS

Lactate dehydrogenase assays

LDH activity was measured in the same blood samples as those used for miRNA measurements, using the enzymatic method on a Dimension RxL Max bioanalyser (Siemens, Saint-Denis, France).

Plasma microRNA (miRNA) extraction

Preliminary experiments were performed to compare plasma and serum miRNA extraction rates and stability. As shown previously (13), the rate of miRNA extraction from plasma was superior to that from serum. In addition, miRNA levels remained constant when plasma was subjected to prolonged storage (> 8 h) at room temperature. Plasma was obtained by standard venipuncture and centrifugation in ethylene-diaminetetraacetic acid (EDTA)-coated tubes. Separation of plasma was performed within 2 h after venipuncture, by centrifugation at 2,400 g for 10 min at ambient temperature. Samples were stored at –80°C.

For microarray analysis and reverse transcription quantitative PCR (RTqPCR) analysis, RNA extraction was carried out using 10 ml and 200 µl of plasma, respectively, with a miRNeasy extraction kit (Qiagen) according to the manufacturer's instructions, followed by elution in 12 µl and 30 µl of water, respectively.

As a control for RTqPCR analysis, 2 µl of miSPIKE solution (150 nM) (Integrated DNA Technologies), a synthetic oligonucleotide corresponding to a sequence that does not exist in the human genome, was added to the plasma samples before RNA isolation, and measured for each sample.

microRNA array analysis

Total RNA (3 µg) was ligated to a RNA-linker-Cy3-dye using T4 RNA ligase (Ambion) with overnight incubation at 4°C, followed by ethanol precipitation. Subsequently, overnight hybridization of labelled RNA was performed at 54°C with LNA-modified microarray slides (Exiqon, version 7) containing duplicate copies of 576 miRNAs of human origin. After extensive washing, slides were scanned using an Axon 4000B scanner in which the photomultiplier (PMT) settings were adjusted automatically. Microarray images were analysed using the GenePix Pro 6.0 software (Axon Instruments). GenePix Results (GPR) were processed using BioPlot software (http://biopuce.insa-toulouse.fr/; available upon administrator agreement). All flagged spots, or background-subtracted spot intensities with values below 0, were removed from the analysis. The signal values for each spot were then normalized, using the total mean signal value obtained from all spots of the array as 100%.

miRNA expression was considered as significant when fold changes in the normalized means of the miRNA expression between the 14 patients and the 5 controls were ≥ 1.5 or ≤ 0.666 and the p-value was ≤ 0.05 according to the Student's t-test (Table SI[1]). In addition, the validity of the results was tested using the Kolmogorov–Smirnov test, and the statistical distance between candidate biomarkers was calculated.

miRNA expression analysis using the 96.96 dynamic array

Reverse transcription (RT) of total RNAs (4 µl) was performed using the miRCURY Universal cDNA synthesis kit (Exiqon) in a 20 µl reaction volume according to the manufacturer's instructions. Specific target preamplification (STA) of cDNA was performed on 1.25 µl of 1:10 RT product using Master Mix TaqMan® PreAmp (Applied Biosystems) and the pool of miRCURY LNA™ primers (Exiqon) with the following protocol: 1 cycle at 95°C for 10 min, 15 cycles at 95°C for 15 s, and then incubation at 60°C for 4 min. qPCR was carried out using the 96.96 Dynamic Array integrated fluidic circuits (Fluidigm Corporation, CA, USA) following the manufacturer's protocol. Briefly, 1.25 µl of 1:10 STA product was loaded for each sample. After loading STA product, runs were carried out in a BioMark® Instrument for PCR using the TaqMan® Gene Expression Master Mix and miRCURY LNA™ primers with the following protocol: 95°C for 10 min, followed by 30 cycles at 95°C for 10 s and incubation at 60°C for 1 min. Each sample was analysed twice. Data were analysed with Real-Time PCR Analysis Software from the BIOMARK instrument (Fluidigm Corp., CA, USA). Only those miRNA assays with an amplification efficiency between 1.7 and 2, a Cq <29 and specific amplification were retained. The $2^{-\Delta Cq}$ method was used to analyse the relative changes in miRNA expression.

Tissue microRNA extraction

RNA was eluted from the spin column membrane with the ultrapure water supplied in the kit. This ultrapure water was used for all subsequent dilutions of RNA samples. RNA yield and purity were evaluated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific) via absorbance measurements at 260 and 280 nm. The high degree of purity of the isolated RNA was confirmed by ensuring that the A260/A280 ratio was in the range 1.96–2.04 for all samples. Isolated total RNA samples were stored at –80°C before use.

Tissue miRNA expression analysis by RTqPCR using the CFX96 PCR System

RT of 300 ng of total RNA was carried out using the miScript II RT kit (Qiagen) following the manufacturer's instructions with HiSpec buffer. qPCR was carried out using the CFX96 PCR system (Bio-Rad) in a 96-well plate format. Reactions were carried out using 0.5 µl of RT product with the miScript primer assay (Qiagen) and the miScript SYBR Green PCR kit (Qiagen). PCR was performed as follows: 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, 30 s at 55°C and 30 s at 70°C. The data were analysed with Bio-Rad CFX Manager software. The $2^{-\Delta Cq}$ method was used to analyse miRNA expression.

Statistical analysis

Data normalization. Data were analysed with the "R" software and the SlqPCR package (14). As a first step, the miRNAs were ranked according to their stability measure, M. The M values were assessed for all the miRNAs from both cohorts and the miRNA with the highest M value was excluded (selectHKgenes function). M values were then calculated for the remaining miRNAs. This procedure was repeated until 3 reference miRNAs were selected. Data were then normalized by geometric averaging of the 3 reference miRNAs using the normPCR function.

When the validation cohort was evaluated, 7 individuals from the training cohort (Fig. 1) were studied again; thus 2 sets of measurements were available for these individuals. As we expected to obtain the same results from both sets of measurements, the difference between the 2 sets was seen as an estimate of the drift resulting from uncontrolled parameters. This estimate was used as a correction value and applied to the measurements of the validation cohort. For all miRNAs, the difference between the mean for the validation cohort and that for the training cohort was calculated. This value was then subtracted from that for the miRNAs of the validation cohort.

Thus, the mean of the values obtained for the repeated individuals was the same for the 2 cohorts.

Data analysis. Bivariate Wilcoxon tests were used to test differences between populations. Kaplan–Meier curves, which estimate the survival rates according to time, were used for univariate survival analysis. Kaplan–Meier curves require a categorical classification of patients, so when the data were continuous they were split around the median. The log-rank test was used to determine whether the survival rates in the groups were different. For multivariate survival analysis, a Cox proportional hazard model was used. For all the methods, a test was considered significant if the *p*-value was < 5%. The tests were carried out for description or variable selection purposes, therefore no multiplicity correction was carried out; to eliminate the false discovery risk, a validation phase was performed. The random forest algorithm (R package random Forest, (15)), which operates by constructing several decision trees, was used as the classification method for analysing the data and for generating the rules of assignment. The random forest algorithm was also used to rank the miRNAs with the Gini accuracy index, using variable importance (Vi). The most relevant number of variables to use in the model, namely k, was determined using a nested cross-validation procedure. The k variables with the highest Vi were selected.