Bone & Joint Open

Supplementary Material

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Venous blood sampling and RNA extraction from EDTA plasma samples

In terms of venous blood sampling, two serum tubes with approximately two ml of venous blood were drawn each and coagulated at room temperature for ten to 30 minutes. Subsequently, the tubes were centrifuged at 15,000 reads assigned per million (rpm) for ten minutes and then stored immediately at -80°C until further processing.

The miRNeasy Mini Kit (Qiagen, Germany) was used to perform RNA extraction. Upon RNA isolation, frozen ethylenediaminetetraacetic acid (EDTA) plasma samples at -80°C were thawed on ice and centrifuged at 12,000 rpm for five minutes. After centrifugation, 200 µl of serum was mixed with 1,000 µl Qiazol and 1 µl of a mix of three synthetic spike-in controls (RNA Spike-In Kit, Qiagen). The mixtures were incubated at room temperature for 15 minutes, and subsequently 200 µl chloroform was added to the lysates followed by further centrifugation at 12,000 rpm for another 15 minutes at +4°C. Exactly 650 µl of upper aqueous phase was mixed with 7 µl glycogen (5 mg/ml). Samples were transferred to a miRNeasy mini-column where RNA was precipitated with 750 µl ethanol and further processed using QiaCube liquid-handling robot. Finally, total RNA was eluted in 30 µl nuclease-free water and stored at -80°C until further analysis.

Tissue sample collection and RNA isolation

Tissue samples with a size of 5 mm x 5 mm were harvested from the periprosthetic tissue and were directly placed in a test tube filled with RNAlater (Qiagen) and stored at -80°C for subsequent analysis. During the analysis, tissue samples were homogenized using FastPrep (MP Biomedicals, USA). Subsequently, RNA extraction was performed with miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, using glycogen to enhance RNA yield (see RNA extraction from EDTA plasma samples). Finally, total RNA was eluted in 30 µl nuclease-free water and RNA quality control was performed using Agilent Bioanalyzer RNA 6000 Nano Assay (Agilent, USA). RNA was stored at -80°C until further analysis.

Small RNA sequencing analysis

For plasma samples, 8.5 µl total RNA was used as an input for small RNA sequencing library preparation. For tissue samples, 100 ng RNA was used as input for library preparation.

To each RNA sample, 1 µl of mind spike-in standards (TAmiRNA, Austria) was added prior to small RNA library preparation using RealSeq Biofluids library preparation kit (RealSeq Biosciences, USA). Adapter-ligated libraries were amplified with 20 polymerase chain reaction (PCR) cycles for plasma and 18 cycles for tissue using both barcoded Illumina reverse and forward primers. Library quality control was performed using DNA1000 Chip (Agilent). Am equimolar pool consisting of all sequencing libraries was prepared and sequenced on an Illumina NovaSEq SP Flowcell in SR100 mode.

Next-generation sequencing (NGS) data were processed using the miND pipeline: after demultiplexing the overall quality of NGS data, it was evaluated automatically and manually with

fastQC v0.11.8 and multiQC v1.7. Reads from all passing samples were adapter-trimmed and quality-filtered using cutadapt v2.3 and filtered for a minimum length of 17nt. Mapping steps were performed with bowtie v1.2.2 and miRDeep2 v2.0.1.2, whereas reads were mapped first against the genomic reference GRCh38.p12 provided by Ensembl allowing for two mismatches and subsequently miRBase v22.1, filtered for miRNAs of hsa only, allowing for one mismatch. For a general RNA composition overview, non-miRNA mapped reads were mapped against RNAcentral and then assigned to various RNA species of interest.

Statistical analysis of pre-processed NGS data

Statistical analysis of pre-processed NGS data was done with R v3.6 and the packages pheatmap v1.0.12, pcaMethods v1.78, and genefilter v1.68. Differential expression analysis with edgeR v3.28 used the quasi-likelihood negative binomial generalized log-linear model functions provided by the package. The independent filtering method of DESeq2 was adapted for use with edgeR to remove low abundant miRNAs and thus optimize the false discovery rate correction of p-values.

Data analysis for heatmap visualization

Data was presented using heatmaps based on rpm normalized reads and scaled using the unit variance method for visualization. Clustering was done using the average method of pheatmap calculating the distances as correlations.

Table i. All detected circulating micro RNAs with significant association to CRP levels.

miRNA	Estimate (r)	p-value	p.adj.
hsa-miR-331-5p	0.7675	0.0001	0.0017
hsa-miR-589-5p	0.7299	0.0003	0.0039
hsa-miR-584-5p	0.7286	0.0003	0.004
hsa-miR-502-3p	0.7182	0.0004	0.0042
hsa-miR-1306-5p	0.7091	0.0005	0.005
hsa-miR-362-3p	0.6909	0.0007	0.0074
hsa-miR-3605-3p	0.6831	0.0009	0.0083
hsa-miR-454-5p	0.6792	0.001	0.0087
hsa-miR-542-3p	0.6792	0.001	0.0087
hsa-miR-424-3p	0.6779	0.001	0.0088
hsa-miR-296-5p	0.6753	0.0011	0.009
hsa-let-7a-3p	0.6675	0.0013	0.0103
hsa-miR-425-3p	0.6545	0.0017	0.0121
hsa-miR-484	0.6545	0.0017	0.0121
hsa-miR-1290	0.6532	0.0017	0.0122
hsa-miR-148b-3p	0.6532	0.0017	0.0122
hsa-miR-542-5p	0.6481	0.0019	0.0134
hsa-miR-423-5p	0.6429	0.0021	0.0143
hsa-miR-505-3p	0.6377	0.0024	0.0155
hsa-miR-629-5p	0.6351	0.0025	0.016
hsa-miR-532-5p	0.6338	0.0026	0.0162
hsa-miR-148a-3p	0.6312	0.0027	0.0165
hsa-miR-3615	0.6312	0.0027	0.0165
hsa-miR-500a-3p	0.626	0.003	0.0174
hsa-miR-7847-3p	0.626	0.003	0.0174

hsa-miR-4454	0.6247	0.003	0.0177
hsa-miR-210-3p	0.6208	0.0033	0.0185
hsa-miR-1294	0.6195	0.0034	0.0188
hsa-miR-22-3p	0.6091	0.0041	0.0217
hsa-miR-3960	0.6091	0.0041	0.0217
hsa-miR-574-5p	0.6078	0.0042	0.022
hsa-miR-6513-3p	0.6	0.0048	0.0238
hsa-miR-1908-5p	0.5987	0.0049	0.0242
hsa-miR-21-3p	0.5909	0.0056	0.0269
hsa-miR-128-3p	0.5883	0.0059	0.0279
hsa-miR-421	0.5883	0.0059	0.0279
hsa-miR-6511a-3p	0.5844	0.0063	0.0293
hsa-miR-1180-3p	0.5818	0.0066	0.0303
hsa-miR-451a	-0.5779	0.007	0.0321
hsa-miR-660-5p	0.5753	0.0073	0.033
hsa-miR-877-5p	0.5727	0.0076	0.0341
hsa-miR-92b-3p	0.5727	0.0076	0.0341
hsa-miR-598-3p	0.5714	0.0078	0.0346
hsa-miR-188-5p	0.5701	0.0079	0.0348
hsa-miR-197-3p	0.5701	0.0079	0.0348
hsa-miR-338-5p	0.5675	0.0083	0.0354
hsa-miR-574-3p	0.5675	0.0083	0.0354
hsa-miR-550a-3-5p	0.5662	0.0085	0.0357
hsa-let-7e-5p	0.5649	0.0086	0.0358
hsa-miR-151a-3p	0.5649	0.0086	0.0358
hsa-miR-96-5p	0.5649	0.0086	0.0358
hsa-miR-106a-3p	0.561	0.0092	0.0379
hsa-miR-27a-3p	0.5597	0.0094	0.0385
hsa-miR-1287-5p	0.5584	0.0096	0.0391
hsa-miR-101-5p	0.5571	0.0098	0.0394
hsa-miR-17-3p	0.5558	0.01	0.0398
hsa-miR-550a-5p	0.5558	0.01	0.0398
hsa-miR-24-3p	0.5545	0.0102	0.0401
hsa-miR-15b-3p	0.5532	0.0104	0.0405
hsa-miR-548aj-5p	0.5506	0.0108	0.0414
hsa-miR-548g-5p	0.5506	0.0108	0.0414
hsa-miR-548x-5p	0.5506	0.0108	0.0414
hsa-miR-1260a	0.5494	0.011	0.0415
hsa-miR-2110	0.5494	0.011	0.0415
hsa-miR-30c-5p	0.5481	0.0112	0.0421
hsa-miR-23b-3p	0.5468	0.0115	0.0426
hsa-miR-548ar-5p	0.5468	0.0115	0.0426
hsa-miR-1285-3p	0.5442	0.0119	0.0437
hsa-miR-25-3p	0.5442	0.0119	0.0437
hsa-miR-660-3p	0.5403	0.0126	0.0452
hsa-miR-1273h-5p	0.5377	0.0131	0.0458
hsa-miR-223-3p	0.5338	0.0139	0.0474
hsa-miR-3928-3p	0.5338	0.0139	0.0474

hsa-miR-548am-5p	0.5338	0.0139	0.0474
hsa-miR-548c-5p	0.5338	0.0139	0.0474
hsa-miR-548o-5p	0.5338	0.0139	0.0474

Table ii. All up- and downregulated micro RNAs in the tissue samples of the high-grade group in comparison to the aseptic group.

miRNA	log2FC	logCPM	F	p-value	FDR
hsa-miR-376a-2-5p	2	2.9	29	0.000045	0.014
hsa-miR-1185-5p	1.4	4.4	28	0.000063	0.014
hsa-miR-337-3p	1.6	4.5	26	0.00009	0.014
hsa-miR-299-5p	1.9	4.6	25	0.00011	0.014
hsa-miR-377-3p	1.5	6.3	21	0.00024	0.017
hsa-miR-224-5p	2.1	8.3	21	0.00026	0.017
hsa-miR-2110	-1.7	4.9	20	0.00031	0.017
hsa-miR-329-3p	1.3	3	19	0.00038	0.017
hsa-miR-542-5p	2.1	4.1	19	0.0004	0.017
hsa-miR-376a-5p	1.4	4.6	19	0.00042	0.017
hsa-miR-424-5p	2.1	7.3	19	0.00043	0.017
hsa-miR-369-3p	1.4	7	19	0.00043	0.017
hsa-miR-543	1.5	6.2	19	0.00045	0.017
hsa-miR-370-5p	1.2	2.5	18	0.00049	0.017
hsa-miR-376c-3p	1.2	10	18	0.00054	0.018
hsa-miR-485-3p	1.7	4.5	17	0.00067	0.021
hsa-miR-487a-3p	1.5	3.5	17	0.00071	0.021
hsa-miR-154-3p	1.7	4.7	16	0.00091	0.025
hsa-miR-542-3p	1.8	5.8	16	0.00096	0.025
hsa-miR-493-5p	1.8	4.9	14	0.0016	0.041
hsa-miR-495-3p	1.6	3.3	14	0.0017	0.041
hsa-miR-376a-3p	1.2	9.4	14	0.0019	0.041
hsa-miR-656-3p	1.4	3.9	13	0.0019	0.041
hsa-miR-382-3p	1.2	5.9	13	0.0024	0.05
hsa-miR-3200-3p	-1.5	4.4	13	0.0025	0.05

CPM, counts per million; FDR, false discovery rate.