miRNA microarray reveals specific expression in the peripheral blood of glioblastoma patients

LUN DONG[1], YUPING LI[1], CHONGXU HAN[2], XIAODONG WANG[1], LEI SHE[1] and HENGZHU ZHANG[1]

Departments of [1]Neurosurgery and [2]Central Laboratory, Clinical Medical College of Yangzhou University, Yangzhou, Jiangsu 225001, P.R. China

Received February 14, 2014; Accepted April 17, 2014

DOI: 10.3892/ijo.2014.2459

Abstract. MicroRNAs (miRNAs) are frequently dysregulated in glioblastoma (GBM) patients. It has been discovered that highly stable extracellular miRNAs circulate in the blood of both healthy individuals and patients. miRNAs in serum of patients with GBM and normal controls were analyzed by microarray analysis. The relevant bioinformatic analysis of the predicted target genes (gene ontology, pathway, gene network analysis) were performed. The miRNA microarray reveals differentially expressed miRNAs in serum between the GBM and normal controls. Of the 752 miRNAs, 115 miRNAs were upregulated in the GBM group, and 24 miRNAs were down-regulated (fold change ≥2.0, P<0.01). By further analysis, we found that miR-576-5p, miR-340 and miR-626 were significantly overexpressed, but miR-320, let-7g-5p and miR-7-5P showed significantly low expression in GBM patients. By further bioinformatic analysis, we found that they possibly play important roles in the regulation of glioma signaling pathways. In summary, the six miRNAs are significant distinct in the peripheral blood of patients with GBM pathologies. These data suggest that the miRNA profile of the peripheral blood may serve as a new biomarker for glioma diagnosis with high specificity and sensitivity.

Introduction

GBM is the most common and aggressive primary brain tumor with high mortality and morbidity. The prognosis for malignant gliomas has not significantly improved in the last four decades (1,2). In the past two decades, the molecular mechanisms, genetics and pathways to treat GBM have extensively been studied. However, the precise mechanism of GBM is unknown and its median survival rate is very low (3,4). The early detection and assessment of GBM pathologies still need to be solved.

MicroRNAs are 21-25 nucleotide small, non-coding RNAs that post-transcriptionally repress the expression of protein-coding genes through binding to the 3' untranslated regions (UTR) of the target mRNAs (5-9). Accumulated evidence indicates that miRNAs are important in the regulation of many biological processes, such as developmental timing, cell metabolism, cell differentiation, cell death, cell proliferation, haematopoiesis and patterning of the nervous system (10). In the past several years, the importance of microRNAs (miRNAs) in cancer cells has been recognized. Proper control of miRNA expression is essential for maintaining a steady state. The stenoplastic existence of circulating miRNAs in the blood of cancer patients has raised the possibility that miRNAs may serve as a novel diagnostic marker (11).

In this study, we profiled miRNAs expression in the peripheral blood of GBM and healthy humans by microarray. The differentially expressed miRNAs were analyzed, then selected to bioinformatic analyses of target prediction. The predicted target genes were subjected to bioinformatic analyses, including gene ontology and pathway analyses. Analyzing the potential molecular markers and the possible relationship between the differentially expressed protein-coding genes and miRNAs in periopheral blood of GBM will help to give further insight into the pathogenesis of GBM.

Materials and methods

Sample preparation and RNA extraction. Selection of subjects, study design and blood sampling. The miRNA profiles from 3 circulating blood samples of GBM patients and 3 age- and gender-matched healthy controls from donors without GBM were obtained according to clinical protocols at the clinical medical college of Yangzhou University. All subjects were generally in good health, and none had diabetes or any other serious concomitant diseases. The patients had not received prior treatment. Following informed consent at the time of acquisition, the samples were collected, and stored at liquid nitrogen. Blood for plasma preparation was collected using a 19-gauge needle into vacutainers containing 0.129 M sodium citrate (1 volume anticoagulant and 9 volumes whole blood) as anticoagulant, centrifuged at 2000 g for 15 min at 22˚C,

Correspondence to: Dr Hengzhu Zhang, Department of Neurosurgery, Clinical Medical College of Yangzhou University, Yangzhou, Jiangsu 225001, P.R. China
E-mail: lundongu571@163.com

Key words: glioma, microRNA, microarray, pathway, blood

transferred into sterile cryovials in aliquots of 1 ml and stored at -70˚C until further analysis. RNA extractions from plasma and all qPCRs were performed by Exiqon Services (Vedbaek, Denmark). Total-RNA was extracted from plasma using miRNeasy Mini kits. Plasma was thawed on ice and centrifuged (1000 x g; 5 min; 4˚C). Plasma (200 μl) was mixed with 750 μl of Qiazol containing 0.94 μg/μl of MS2-bacteriophage (incubated for 5 min, room temperature). Samples were chloroform extracted, ethanol precipitated, transferred to RNeasy Mini columns and washed according to the manufacturer's protocols. RNA was eluted in 50 μl RNase-free water.

MicroRNA real-time PCR array. Human panelI+II v3 (the 752 micRNAs) miRCURY LNA™ Universal RT miRNA PCR array were obtained from the manufacturers. The qRT-PCR-based platforms promise to be more sensitive than array-based miRNA quantification platforms (12), and their use for analyzing samples with low miRNA levels, such as human plasma, is increasing (13-18).

RNA was reversely transcribed using miRCURY LNA Universal RT microRNA PCR, polyadenylation and cDNA synthesis kit (Exiqon, Vedbaek, Denmark). PCR reactions (10 μl) were performed according to the miRCURY LNA Universal RT microRNA PCR protocol; each miRNA was assayed once on microRNA Ready-to-Use PCR Human panel I+II (Exiqon, Kangchen, China). Controls included primers for six independent reference genes, including 3 microRNAs (hsa-miR-103, hsa-miR-191 and hsa-miR-423-5p), and 3 small RNA (U6, SNORD38B and SNORD49A).

The amplification profile was denatured at 95.8˚C for 10 min, followed by 40 cycles of 95.8˚C for 10 sec and 60.8˚C for 60 sec. At the end of the PCR cycles, melting curve analyses were performed. All reactions were done in triplicate. Amplification efficiency was calculated using algorithms similar to LinReg (Exiqon, Vedbaek, Denmark). All assays were inspected for distinct melting curves and melting temperatures were checked to be within known assay specifications. Expression levels of mature mRNAs were evaluated using comparative CT method ($2^{-\Delta CT}$) (19). Raw Ct values were calculated as recommended by Exiqon Negative controls, without enzymes, from the reverse transcription reaction was treated and profiled. Data that did not pass these criteria were omitted from any further analysis.

Statistical analysis. The approximate normal distribution of the measured data was verified by Shapiro-Wilk test. Data are expressed as the mean ± SD unless otherwise noted. The differences between groups were analyzed using by unpaired two-tailed parametric t-test when only two groups were present and the null hypothesis was rejected at the 0.05 level. The correlation between two dichotomous variables was assessed using Fisher's exact test. The enrichment P-value of the PathwayID used Fisher's exact test.

Results

Differentially expressed miRNAs in glioma patients. Altogether, 752 mature miRNAs were quantitatively analyzed using a microarray platform. Following background subtraction and quantile normalization, all miRNAs with median intensity below 100 were considered lowly abundant and removed. The remaining 139 miRNAs (fold change ≥2.0) were then analyzed using computational approaches (Fig. 1). Compared to peripheral blood of control cases, we observed significant deregulation of 139 miRNAs, 115 miRNAs were upregulated (83%) (Table I), whereas 24 miRNAs were downregulated (17%) (Table II). Especially three miRNAs, the miR-576-5p, miR-340, miR-626, the most upregulated and miR-320, let-7g-5p, miR-7-5P, the most downregulated miRNA, in glioma patients. Of the six miRNAs, miR-576-5p displayed a 3.05-fold increased median expression in samples from glioblastoma patients compared to healthy controls. The expression level of miR-7-5P was decreased in the glioblastoma patients to 0.37-fold compared with the healthy donors.

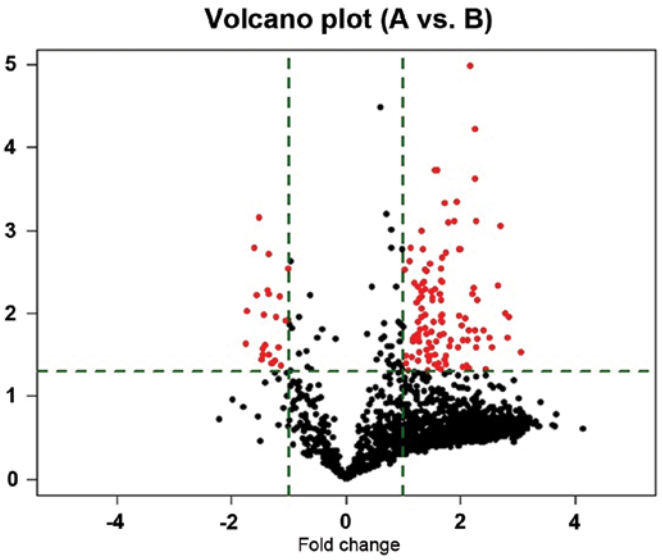

Figure 1. Scatter plot comparison of the fold changes of microarray experiments. Scatter plots comparing non-normalized signal intensities of miRNAs between the GBM sample and the normal brain sample from human total serum. Red and black denotes high and low miRNA expressions, respectively. Comparison of the fold changes of 139 miRNAs found significantly deregulated in gliomas patients in the microarray experiments and the corresponding qRT-PCR results.

Prediction of targets of differentially expressed miRNA and function annotation in peripheral blood of GBM patients. To understand the potential functions of significantly differentially expressed miRNA in the GBM, and to further explore the function of these predicted target genes, we selected the most upregulated coherent 12 miRNAs (hsa-miR-4726-5p, hsa-miR-1255b-2-3p, hsa-miR-340-5p, hsa-miR-4275, hsa-miR-4712-3p, hsa-miR-576-5p, hsa-miR-1299, hsa-miR-4268, hsa-miR-3591-5p, hsa-miR-626, hsa-miR-BART18-3p, hsa-miR-3169), and the most downregulated coherent 8 miRNAs (hsa-miR-320b, hsa-let-7g-5p, hsa-miR-486-5p, hsa-miR-7-5p, hsa-miR-4524b-5p, hsa-miR-3171, hsa-miR-1246, hsa-miR-1273g-3p) to perform Gene Ontology analysis and pathway analysis.

To understand the potential functions of significantly differentially expressed miRNA in glioma patients, various biological process terms of genes were predicted as the potential targets of these 20 miRNAs in the peripheral blood

Table I. Significant markers for differentiation of A vs. B 2.0-fold downregulated miRNAs.

ID	Name	Fold change A vs. B	P-value A vs. B	Mean of B group	Mean of A group	CV-value B group	CV-value A group
146190	hsa-miR-3927-3p	3.203477534	0.012777	10.33333	30.66667	0.30381	0.261223
148599	hsa-miR-3680-5p	3.051520614	0.039572	56.66667	171.6667	0.294048	0.37474
148206	hsa-miR-3664-5p	2.517210786	0.001675	64.66667	157.5	0.249211	0.097353
169031	hsa-miR-4726-5p	2.346233498	0.018606	753	1731	0.194819	0.245785
46924	hsa-miR-1252	3.271682269	0.035915	22.83333	67.16667	0.425179	0.364375
168954	hsa-miR-5580-5p	3.283873506	0.036384	23.83333	75	0.146777	0.38659
168811	hsa-miR-1255b-2-3p	2.991009789	0.000186	136.5	390	0.080275	0.082555
10138	hsa-miR-130a-3p	2.578573273	0.002984	18.66667	45.33333	0.293111	0.118974
148640	hsa-miR-34b-3p	5.831082399	0.025291	5.333333	30.83333	0.200898	0.410603
169132	hsa-miR-382-3p	8.247805209	0.029701	3.166667	27.5	0.700007	0.452172
169064	hsa-miR-4778-3p	2.806551133	0.025484	39.33333	100.6667	0.438363	0.280325
42744	hsa-miR-23a-3p	2.726506347	0.022091	16.83333	42.33333	0.602761	0.205527
29872	hsa-miR-340-5p	2.353775226	0.007455	137	300.8333	0.339218	0.137188
11074	hsa-miR-34c-5p	7.088329336	0.010987	5	35.16667	0.263536	0.329962
146085	hsa-miR-3170	4.894704669	0.020575	6	28.5	0.03643	0.370965
145643	hsa-miR-382-5p	4.369951187	0.016087	10.83333	45.5	0.109142	0.332717
147831	kshv-miR-K12-1-3p	2.659428299	0.032152	27.5	71.66667	0.11655	0.332325
147743	hsa-miR-4275	3.889772826	0.001654	66	246.3333	0.03748	0.170299
148593	hsa-miR-3605-3p	3.070344638	0.045766	18	52.5	0.244884	0.39999
148420	hsa-miR-3607-3p	4.329000571	0.020676	8.166667	34	0.094569	0.358471
148382	hsa-miR-3609	4.069918082	0.044562	5.833333	23	0.215226	0.448962
148215	hsa-miR-3591-3p	2.77311974	0.002476	37.83333	99	0.107459	0.158783
168730	hsa-miR-4464	3.109959132	0.005786	8.166667	24	0.170293	0.211635
168644	hsa-miR-4775	2.653797224	0.017591	17.83333	45	0.154669	0.2708
168671	hsa-miR-3140-5p	2.73656565	0.032136	31	77.16667	0.352385	0.315583
168917	hsa-miR-4511	3.154343491	0.002866	77.16667	223.3333	0.440141	0.116191
169023	hsa-miR-4712-3p	2.898882114	0.00506	113.5	300.1667	0.348018	0.164139
169247	hsa-miR-4477a	2.066080045	0.032175	26.16667	50.66667	0.273629	0.243579
169407	hsa-miR-4301	3.147673634	0.006956	37	109.6667	0.137091	0.227335
148241	hsa-miR-3649	2.013497623	0.002943	22	42.33333	0.068752	0.13039
42446	hsa-miR-576-5p	3.184821155	0.004122	176.1667	537.3333	0.289242	0.179645
145852	hsa-miR-210	2.258332188	0.020024	29.66667	60.66667	0.572146	0.04695
147614	hsa-miR-4299	3.136510824	0.047028	10.83333	32.66667	0.112652	0.414372
168727	hsa-miR-4426	2.423405402	0.029561	100.1667	220.3333	0.503972	0.225886
169138	hsa-miR-4504	2.383728075	0.019578	18.33333	41.33333	0.223376	0.24957
42576	hsa-miR-342-5p	2.843331991	0.006531	24.5	63.16667	0.570785	0.079951
19588	hsa-miR-17-3p	3.077276567	0.018349	12.33333	34.83333	0.515709	0.253543
10995	hsa-miR-199a-3p/ hsa-miR-199b-3p	4.88116897	0.006986	7.333333	34.33333	0.096524	0.269352
46788	hsa-miR-1299	2.473827682	0.008765	215.3333	486.8333	0.436409	0.124299
11022	hsa-miR-221-3p	2.427402229	0.006613	30.5	68.66667	0.326156	0.143626
10936	hsa-miR-130b-3p	2.067075639	0.040137	35.16667	65.66667	0.592687	0.083123
147809	hsa-miR-514b-3p	2.579816857	0.014999	13.66667	33.33333	0.233535	0.243151
147805	hsa-miR-3183	3.861577136	0.025355	18.5	64.66667	0.581673	0.336679
148065	hsa-miR-3689b-3p/ hsa-miR-3689c	3.136474117	0.010713	9.5	28	0.292469	0.244212
148402	hsa-miR-3920	4.001086173	0.001655	24.33333	87.83333	0.604129	0.08289
148064	hsa-miR-3926	2.5086651	0.004276	28.33333	64.83333	0.428735	0.050641

Table I. Continued.

ID	Name	Fold change A vs. B	P-value A vs. B	Mean of B group	Mean of A group	CV-value B group	CV-value A group
168796	hsa-miR-3664-3p	2.369986306	0.037827	33.5	74.83333	0.29549	0.30304
169080	hsa-miR-4684-5p	3.284979213	0.000461	12	36.5	0.333739	0.052713
169056	hsa-miR-4669	3.826929416	0.000454	11.83333	41.33333	0.434247	0.042278
169356	hsa-miR-548aa/ hsa-miR-548ap-3p/ hsa-miR-548t-3p	2.460868369	0.015366	29.66667	68	0.36115	0.206501
6880	hsa-miR-297	2.3954973	0.012651	21	47.66667	0.184716	0.221655
169054	hsa-miR-4422	4.445358595	1.04E-05	8	33.33333	0.147536	0.035785
17863	hsa-miR-934	2.361722381	0.004803	23	51.5	0.131753	0.167395
42929	hsa-miR-25-5p	2.671452102	0.0491	16	40.83333	0.372552	0.361894
10998	hsa-miR-19b-3p	2.107251706	0.049556	34.83333	68.83333	0.305903	0.292752
168590	hsa-miR-4520a-5p/ hsa-miR-4520b-5p	2.156306634	0.002379	47.33333	97.16667	0.080863	0.130327
169038	hsa-miR-488-3p	4.769928835	0.000239	9.166667	40	0.461345	0.052185
11184	hsa-miR-99b-5p	7.001061122	0.019742	18.5	115.5	0.512366	0.387807
11093	hsa-miR-379-5p	6.293079862	0.00468	7	40.83333	0.222742	0.253094
42723	hsa-miR-195-3p	2.651388508	0.003052	25.5	63.33333	0.219692	0.14664
10997	hsa-miR-19a-3p	3.889673823	0.01088	10.5	38.5	0.264691	0.278187
146066	hsa-miR-3116	3.178096875	0.002139	12	35	0.39386	0.114116
145722	hsa-miR-520e	3.149410672	0.003969	14.5	42.66667	0.347124	0.164727
147735	hsa-miR-4289	2.916146221	0.000189	16	44.5	0.250692	0.002877
147942	hsa-miR-4268	2.285884624	0.018923	81.66667	170.1667	0.494251	0.136427
147752	hsa-miR-4302	4.791125482	0.025783	5.666667	26.66667	0.159828	0.39453
168682	hsa-miR-4502	3.324822913	0.032542	17.5	52	0.458672	0.350977
168773	hsa-miR-5702	4.677848243	0.00501	6.333333	26.83333	0.601745	0.206642
169200	hsa-miR-4677-5p	4.41894338	0.045959	25.33333	99.16667	0.453865	0.457275
168994	hsa-miR-3591-5p	3.309725618	0.041503	335.5	977.5	0.649707	0.357879
169288	hsa-miR-4730	4.709961044	0.015959	17	72	0.378501	0.330353
10947	hsa-miR-142-3p	2.955428209	0.021391	30	81.33333	0.308113	0.294342
147651	hsa-miR-3123	4.735509368	5.95E-05	14.5	66.5	0.158066	0.069491
147556	hsa-miR-4254	3.428058179	0.000803	25.16667	81.33333	0.182843	0.123553
11073	hsa-miR-34b-5p	2.210146571	0.033651	21.5	45.16667	0.164206	0.289177
11041	hsa-miR-29c-3p	3.158712421	0.012225	7.833333	23.83333	0.093391	0.270955
42490	hsa-miR-505-5p	2.366701641	0.017946	31.16667	69.83333	0.257714	0.234164
17898	hsa-miR-99b-3p	3.226226981	0.01745	19.33333	59.66667	0.131474	0.303247
168914	hsa-miR-5689	2.403696329	0.021583	78.33333	185.6667	0.248793	0.256256
168930	hsa-miR-5582-5p	3.705151644	0.000771	24.66667	90.5	0.339369	0.102201
169369	hsa-miR-4490	6.484947988	0.000887	4.833333	29.66667	0.271291	0.159504
17613	hsa-miR-645	3.527654767	0.02085	15.66667	52.33333	0.342237	0.321159
13178	hsa-miR-18a-3p	4.346091391	0.046762	7.166667	30.33333	0.190132	0.467137
147584	hsa-miR-548t-5p	2.469465274	0.005526	25.66667	58.5	0.329129	0.13442
147187	hsa-miR-215	4.598242139	0.005794	8.333333	36.33333	0.213552	0.247929
145670	hsa-miR-18b-5p	4.204749209	0.011257	6.833333	27.16667	0.242225	0.291091
148168	hsa-miR-3658	6.848703552	0.009947	3.5	23.5	0.188594	0.319594
148265	hsa-miR-3935	2.251023577	0.021583	39	79.66667	0.466669	0.161861
42702	hsa-miR-30c-1-3p	3.329134928	0.001831	13.5	40.83333	0.461954	0.08929
168717	hsa-miR-5193	2.7843813	0.005472	28.83333	73.83333	0.391852	0.14674
168686	hsa-miR-4768-5p	5.676106033	0.019686	30.5	153.3333	0.577846	0.365043

Table I. Continued.

ID	Name	Fold change A vs. B	P-value A vs. B	Mean of B group	Mean of A group	CV-value B group	CV-value A group
168557	hsa-miR-4777-5p	2.479209518	0.010962	179.8333	407	0.356283	0.180226
169068	hsa-miR-513c-3p	5.261154548	0.015784	6.333333	32.16667	0.22337	0.345836
169129	hsa-miR-4284	2.591855924	0.005109	15.33333	36.66667	0.292255	0.154386
169026	hsa-miR-4679	4.811020398	0.000761	10.83333	48.83333	0.29521	0.135146
13137	hsa-miR-518e-5p	2.182115742	0.001646	59.16667	126	0.21226	0.077262
148620	hsa-miR-454-3p	2.768247278	0.032807	11.33333	30.16667	0.128874	0.342265
148678	hsa-miR-301a-5p	2.467515688	0.000997	27.66667	63.66667	0.252293	0.06196
168792	hsa-miR-4434	2.817438302	0.006932	11.83333	31.66667	0.160738	0.211055
17668	hsa-miR-552	2.694699994	0.037546	12.33333	32	0.098735	0.353729
13143	hsa-miR-301a-3p	4.00378766	0.014095	6.166667	23.66667	0.148628	0.309825
17546	hsa-miR-585	2.894857265	0.0128	13.33333	36.33333	0.190016	0.256328
42475	hsa-miR-221-5p	2.905359198	0.041188	31.16667	87.66667	0.113936	0.38058
33407	hsa-miR-626	2.580059334	0.010429	157	376.6667	0.221173	0.216872
17636	ebv-miR-BART20-5p	5.387250347	0.047521	3.333333	18.33333	0.477962	0.491119
42466	ebv-miR-BART18-3p	2.274976623	0.004247	80	168.6667	0.23888	0.128316
17870	hsa-miR-628-5p	4.270594044	0.042687	5.333333	22.66667	0.188859	0.450096
147792	hsa-miR-3165	2.497683723	0.006189	18.66667	44	0.20665	0.17864
148624	hsa-miR-942	2.79071413	0.016599	65	168.5	0.395192	0.241847
148059	hsa-miR-493-5p	3.188942439	0.010476	8.333333	25.5	0.186781	0.255087
168968	hsa-miR-147b	2.917356579	0.031679	24.83333	64.83333	0.45819	0.314305
46439	hsa-miR-1243	2.636787535	0.027878	17.33333	44.33333	0.076252	0.317122
145914	hsa-miR-135b-5p	3.485787586	0.024747	13.33333	46	0.232823	0.345846
169198	hsa-miR-3145-5p	2.561803904	0.004227	27.83333	70.33333	0.335386	0.123721
169072	hsa-miR-3925-3p	4.106901165	0.021254	77.16667	336	0.904449	0.280116

miRNAs showing differential upregulated expression between glioblastoma samples (A) and healthy control (B).

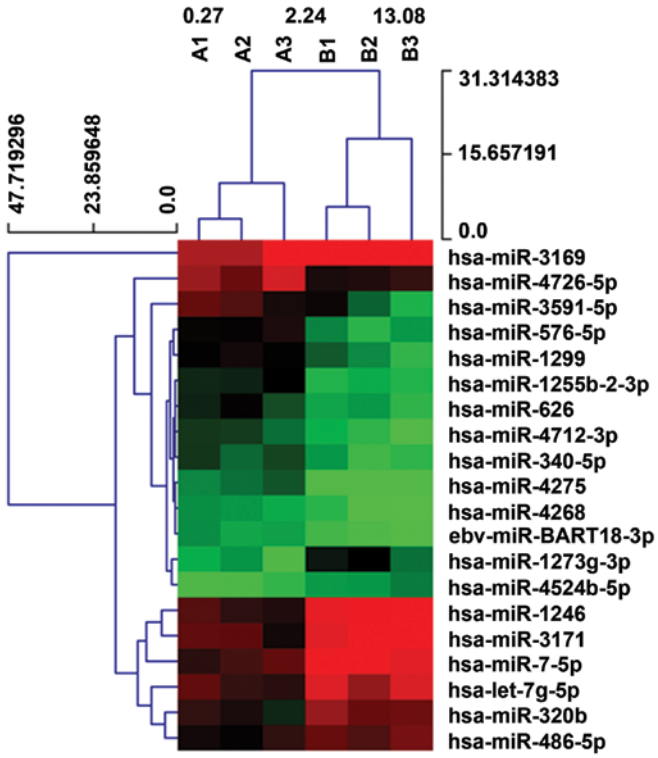

Figure 2. Clustering of the 20 most variable miRNAs. Clustering of the 20 most variable miRNAs for the classification between glioma patients and healthy controls. Complete linkage hierarchical clustering was performed with the Euclidian distance measure. Glioma patients and healthy controls cluster separately. The colors in the heatmap represent the normalized expression values with lower expression values being colored in shades of green and higher expression values in shades of red.

of glioma patients by using bioinformatics method (Fig. 2). To further explore the function of these predicted target genes. The predicted target genes in Gene Ontology (GO) which covers three domains of the 20 miRNAs: biological process, cellular component and molecular function (Fig. 3) biological process terms were enriched with cellular catabolic process and cellular process. In cellular component category, GO terms related to the cytoplasmic part, membrane-bounded organelle, intracellular membrane-bound organelle, organelle, intracellular organelle, cytoplasm and intracellular organelle part. The molecular function category of GO terms showed the major functions. Binding, myosin binding, protein transporter activity, serotonin receptor activity, histone methyltransferase activity, transcription factor binding,

Table II. Significant markers for differentiation of A vs. B 2.0-fold downregulated miRNAs.

ID	Name	Fold change A vs. B	P-value A vs. B	Mean of B group	Mean of A group	CV-value B group	CV-value A group
168640	hsa-miR-4475	0.3938	0.0314	220	84.5	0.320057962	0.114365955
169368	hsa-miR-3529-3p	0.4512	0.0421	256.8333333	105.3333333	0.252745746	0.444514077
147821	hsa-miR-3169	0.3728	0.0237	6285.833333	2340	0.275801006	0.354230901
169308	hsa-miR-4503	0.4398	0.0261	97.16666667	41.33333333	0.264382278	0.219747983
46324	hsa-miR-320b	0.4276	0.0110	1520.666667	599	0.138085914	0.404888839
46438	hsa-let-7g-5p	0.4787	0.0120	2122.166667	957	0.168042032	0.253336484
169182	hsa-miR-4728-3p	0.3001	0.0093	205.8333333	60.16666667	0.149970516	0.70029427
32946	hsa-miR-486-5p	0.4957	0.0116	1316.166667	635	0.123127462	0.313923832
169087	hsa-miR-149-3p	0.3466	0.0006	50.33333333	16.66666667	0.113365131	0.108003899
169255	hsa-miR-4708-5p	0.4236	0.0371	672.5	284	0.303906575	0.270147743
42571	hsa-miR-129-1-3p	0.4064	0.0400	107.8333333	43.16666667	0.294985703	0.430915646
147613	hsa-miR-3145-3p	0.3897	0.0059	120.1666667	44.83333333	0.154426925	0.317036901
42859	hsa-miR-675-3p	0.3379	0.0060	1893.666667	622.1666667	0.211374742	0.136127979
19596	hsa-miR-30d-5p	0.4956	0.0029	693	332.5	0.078739567	0.220298587
169285	hsa-miR-4467	0.3290	0.0016	200.1666667	63.33333333	0.14789289	0.123802767
168892	hsa-miR-4424	0.3574	0.0358	85.5	29.33333333	0.339119811	0.319612141
29490	hsa-miR-7-5p	0.3896	0.0019	2791.666667	1038.5	0.117173492	0.224858694
169137	hsa-miR-4524b-5p	0.4468	0.0062	197.6666667	86.5	0.146863385	0.24004705
11038	hsa-miR-299-5p	0.4080	0.0398	163.1666667	62.83333333	0.319291192	0.296060783
42902	hsa-miR-185-5p	0.3868	0.0052	2599.666667	939.1666667	0.038092839	0.486170761
147952	hsa-miR-3171	0.3716	0.0104	2774.666667	981	0.191177266	0.387816899
168870	hsa-miR-1246	0.2956	0.0230	3013	863	0.330372226	0.275463985
168925	hsa-miR-1273g-3p	0.3623	0.0264	381.5	126.3333333	0.278133806	0.446720161
169143	hsa-miR-4459	0.3663	0.0311	80.66666667	28.33333333	0.257323615	0.593528641
168640	hsa-miR-4475	0.3938	0.0314	220	84.5	0.320057962	0.114365955
169368	hsa-miR-3529-3p	0.4512	0.0421	256.8333333	105.3333333	0.252745746	0.444514077
168640	hsa-miR-4475	0.3938	0.0314	220	84.5	0.320057962	0.114365955
169368	hsa-miR-3529-3p	0.4512	0.0421	256.8333333	105.3333333	0.252745746	0.444514077

miRNAs showing differential downregulated expression between glioblastoma samples (A) and healthy control (B).

transcription factor activity, retinoic acid receptor binding, protein binding transcription factor activity, protein binding, transcription cofactor activity, protein kinase activator activity, acetylglucosaminyltransferase activity. To detect these 20 miRNA pathways, whose members are significantly enriched for targets of the 20 miRNAs being deregulated, we utilized the 'miRNA to pathway dictionary' (20). All the significant target pathways are listed for the miRNA in Fig. 4 and related to pyrimidine metabolism, cytosolic DNA-sensing pathway, serotonergic synapse, base excision repair, calcium signaling pathway, glycosaminoglycan biosynthesis, keratan sulfate, HTLV-I infection and purine metabolism. These have been computed by separately applying standard over-representation analysis for each set of miRNA targets, a gene network analysis was applied to the upregulation of miR-576-5p, miR-340, miR-626 (Fig. 5), and downregulation miR-320, let-7g-5p and miR-7-5P (Fig. 6).

Discussion

Gliomas are the most commonly diagnosed cancers in the brain. Following therapy, progression or recurrence is often observed. In addition to imaging studies, that are normally only available every 2-3 months, biomarkers might be helpful monitoring tools to detect tumor recurrence at the earliest time point or to distinguish between pseudoprogression and substantial tumor growth.

It has been found that a large part of the human genome is transcribed into non-coding RNAs with major functions both in normal physiology and in pathological processes (21,22). Recent studies have shown the contribution of miRNAs to cancer pathogenesis (23). miRNAs have the advantage of being clearly defined markers that can easily be determined by microarrays or real-time PCR. The function of miRNAs appears to be in gene regulation where they act as post-transcriptional regulators and control the target messenger RNAs

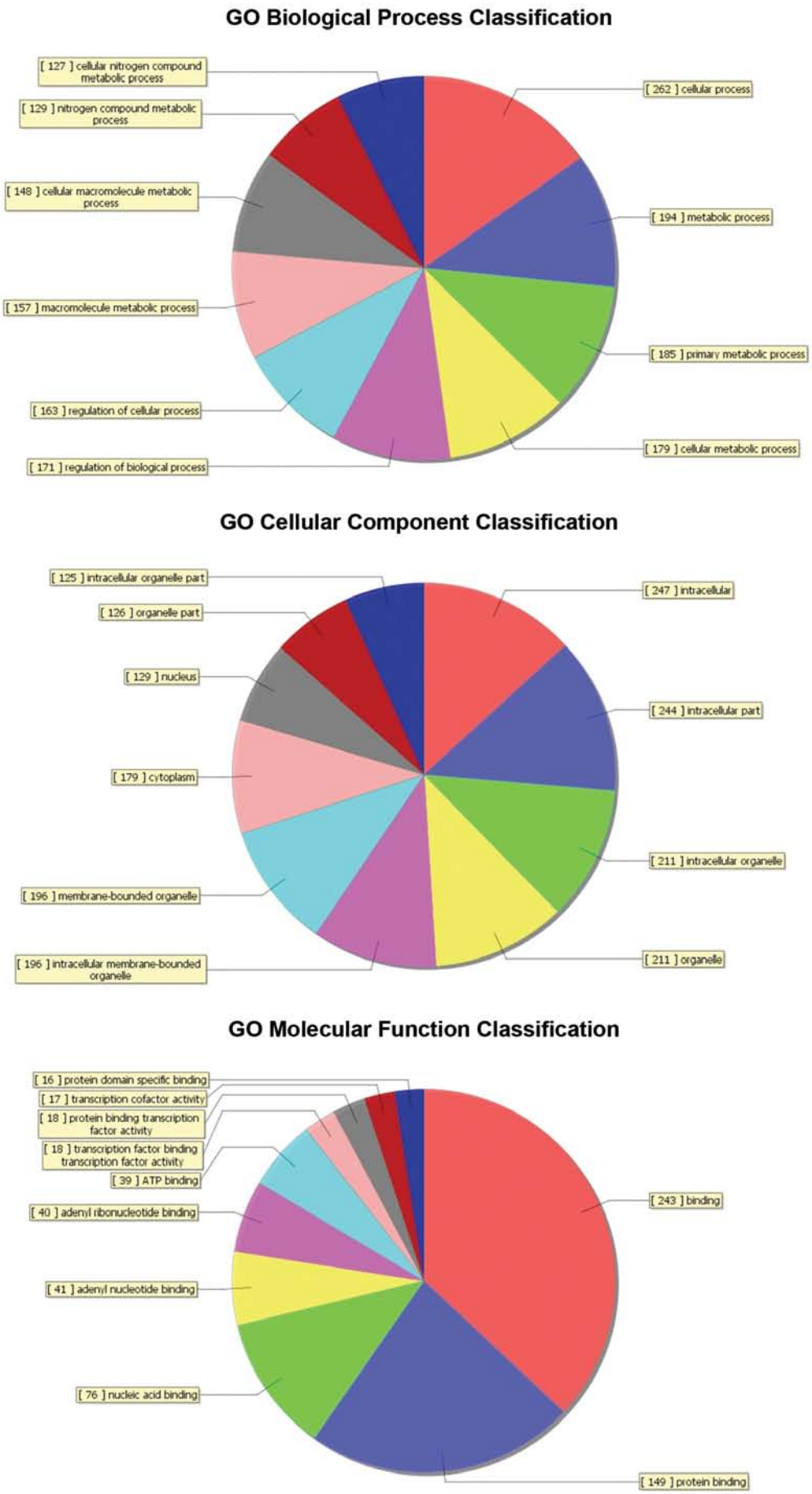

Figure 3. Gene ontology (GO) enrichment analysis for miRNA-targets in the category of biological processes. Gene oncology analysis of miRNA target genes according to biological process, cell component and molecular function.

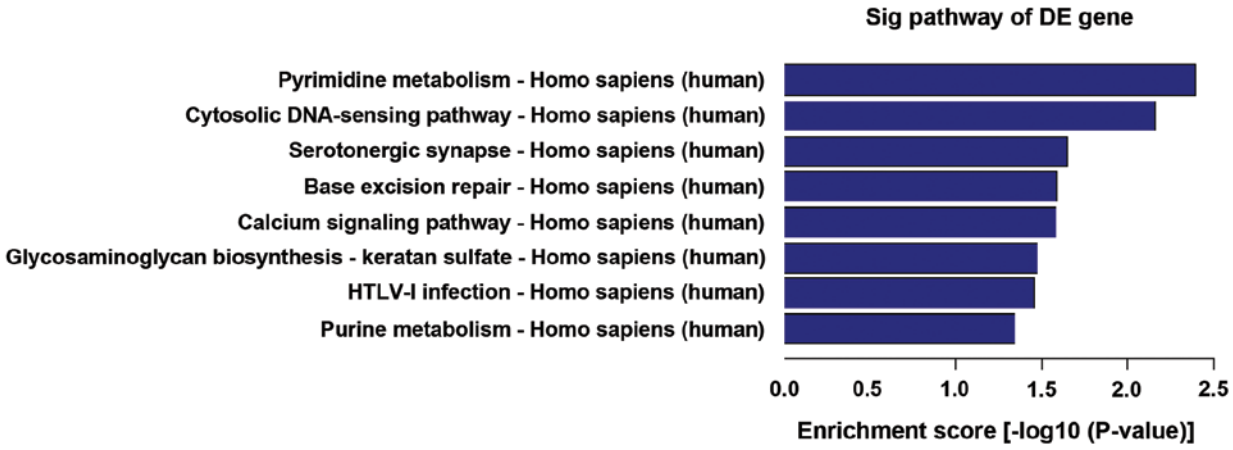

Figure 4. Target gene-related pathways.

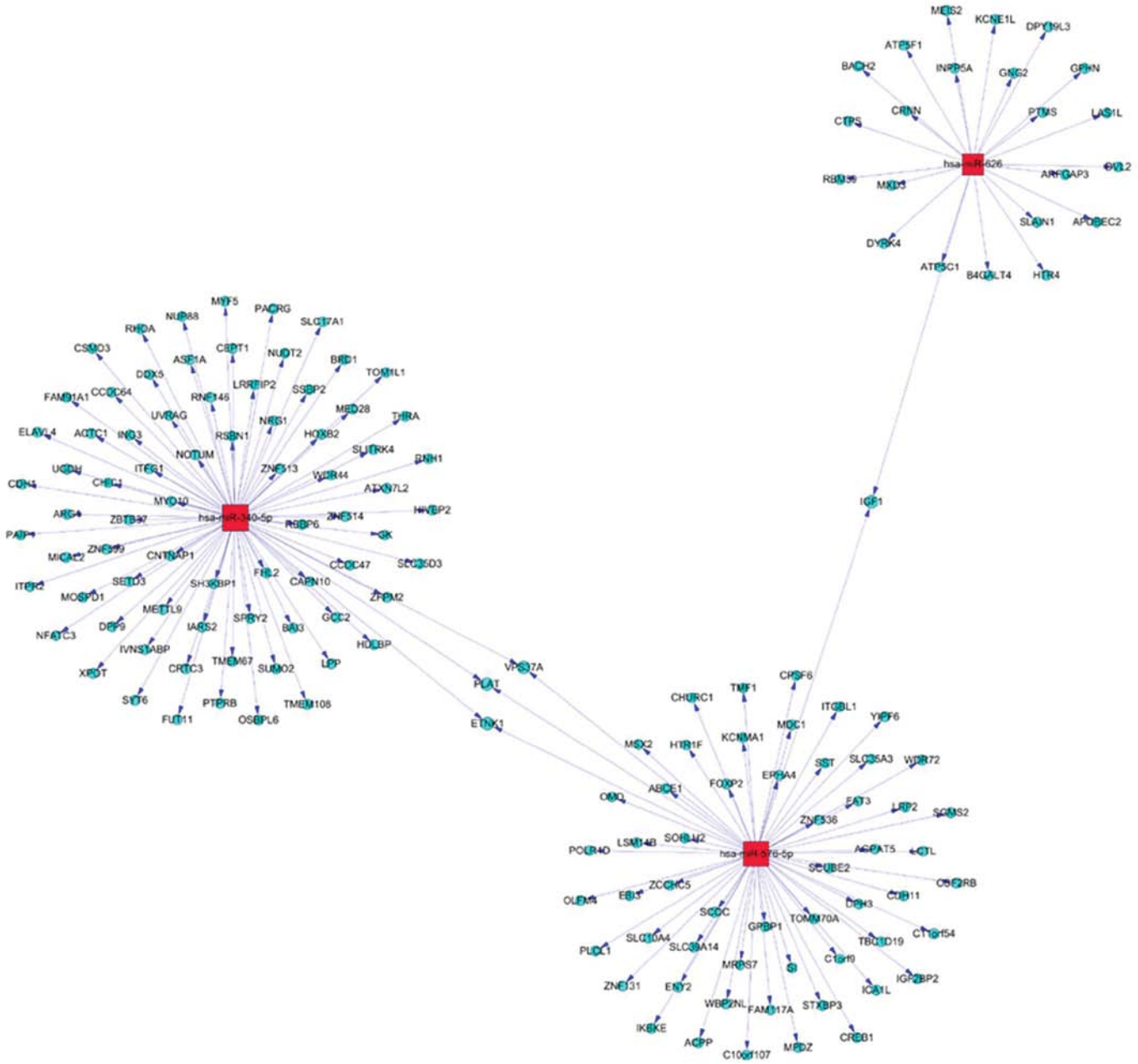

Figure 5. miRNA-mRNA association network. Glioma-related miRNA-gene upregulation network in circulating blood.

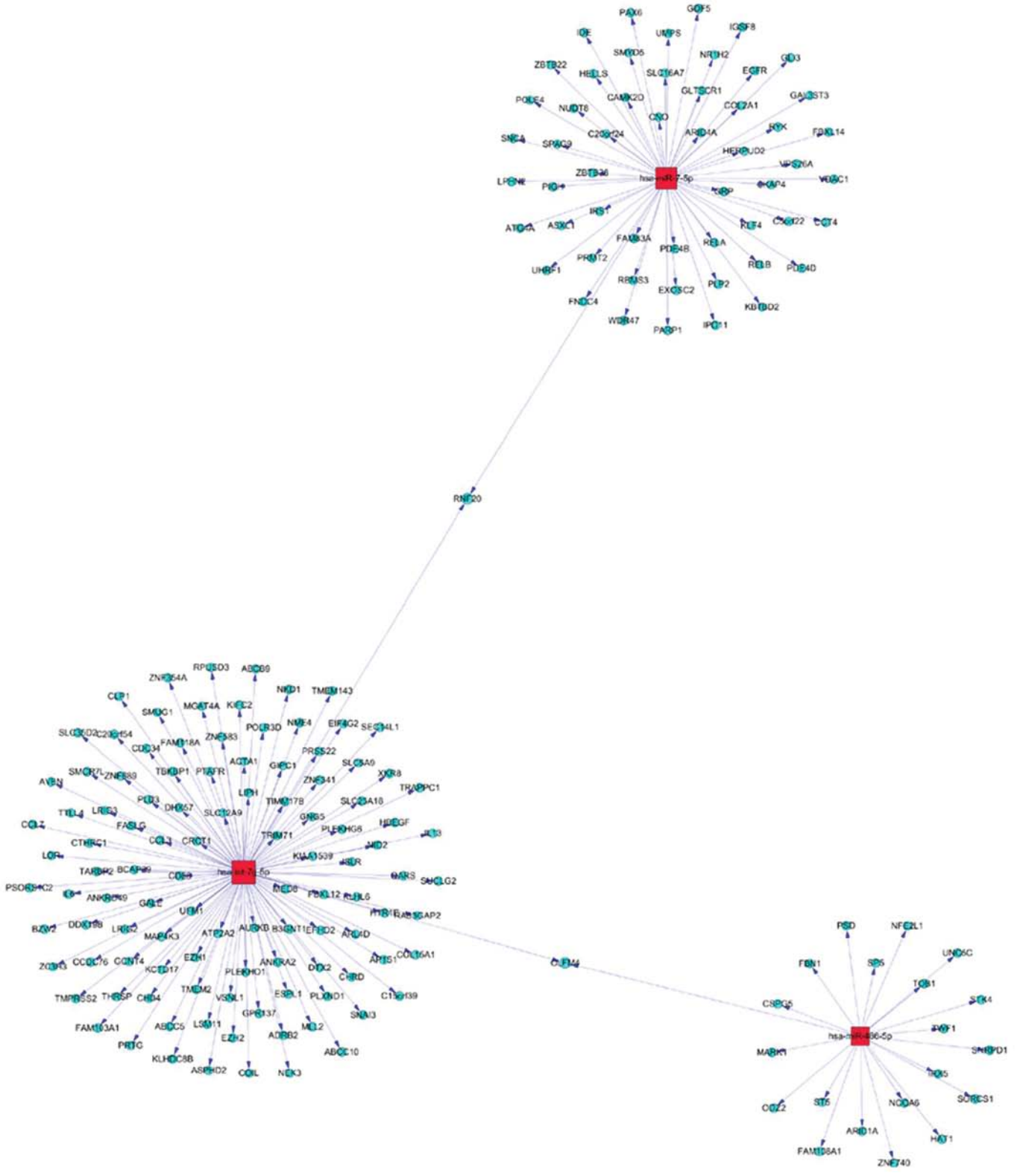

Figure 6. miRNA-mRNA association network. Glioma-related miRNA-gene downregulation network in circulating blood.

(mRNAs). Perfect or near perfect base pairing with the target RNA promotes cleavage and destruction of the mRNA, whereas miRNAs that are only partially complementary to the target may inhibit protein translation of mRNA and also cause the mRNAs to be degraded sooner (24). The mRNA is thus 'silenced' and the protein coded for not produced. miRNAs therefore generally have an 'inhibitory' function (25). In this way, miRNAs appear to have various functions in physiology, from cell differentiation, proliferation and apoptosis to the regulation of the endocrine system and metabolism (12,26). Although the miRNA analysis can be performed by broadly available technologies that are already

in clinical use, but these associations are based on studies in tissue samples. However, such tissue samples are not easily obtained, and a more effective step forward was found when miRNAs were detected to be remarkably stable in serum or plasma (27,28). miRNAs have the advantage of being clearly defined markers that can easily be determined by microarrays or real-time PCR in peripheral blood. The ease of the methods further opens the road for the analysis of specific miRNA patterns that comprise numerous miRNAs.

In this study, initial analysis of the miRNAs profiling showed that some miRNAs were differentially expressed in GBM patients and these miRNAs can be distinguished from normal individuals. We identified 139 miRNAs that were significantly expressed, 115 miRNAs were found to be upregulated whereas 24 were downregulated. Our results were partly in disagreement with Roth *et al* (20). When comparing the filtered repertoire of the 310 miRNAs in glioblastoma patients and healthy controls, they observed a significant deregulation of 52 miRNAs, amounting to 16.8% of analyzed miRNAs. Of these, 27 miRNAs were upregulated (52%) whereas 25 miRNAs were downregulated (48%). The microarray analysis also showed that there was no significantly distinct difference in the 613 common miRNA expression profiles between these two conditions. This could be explained by the fact that both conditions have undergone genetic normalities. It is also probable that both conditions share similar genetic pathways through the regulation of specific gene expression. We identified that miR-576-5p, miR-340, miR-626 and miR-320, let-7g-5p and miR-7-5P were significantly deregulated only in the blood of GBM but not in the blood of normal inviduals, suggesting a possible role in gliomas cell growth and proliferation. For example, miR-342-3p, the second most deregulated miRNA, has not been investigated in glioma tissues. The results of Li *et al* indicated that an overexpression of miR-576-5 occurred in brain-metastatic carcinomas (29), whereas, miR-576-5p was downregulated in osteoarthritis (OA) chondrocyte pellets with the highest fold-change of 4.74-fold (30), but the miR-576-5p was overexpressed in the the serum of glioma patient in this study. A number of recent studies have demonstrated that miR-7 inhibits cell migration and metastasis in glioblastoma (31,32). Recent advances in miRNA delivery and targeting suggest that 'miRNA replacement therapy' with miR-7-5p may be a feasible approach to cancer treatment (33). Giles *et al* demonstrated that miR-7-5p expression is reduced in metastatic melanoma-derived cell lines compared with primary melanoma cells, and that ectopically expressed miR-7-5p significantly inhibits melanoma cell migration and invasion. Additionally, it was reported that insulin receptor substrate-2 (IRS-2) is a target of miR-7-5p in melanoma cells. By using RNA interference (RNAi) evidence was provided that IRS-2 activates protein kinase B (Akt), and promotes melanoma cell migration. Thus, miR-7-5p may represent a novel tumor suppressor miRNA in melanoma, acting at least in part via its inhibition of IRS-2 expression and oncogenic Akt signaling (34). In the present report the miR-7-5p, significantly deregulated miRNA in the peripheral blood of GBM patient, but has not yet been seriously investigated in glioma tissues.

Subsequently, a futher step was added to our study, GO analysis and pathway analysis. The GO analysis included biological process, molecular function and cellular components. Pathway analysis was used to generate networks and assess statistically relevant biofunctions and canonical pathways that predicted target genes involved. These genes were mapped to corresponding genes in the Ingenuity knowledge database. The biofunctional analysis identified the molecular and cellular function, physiological system development and function. Canonical pathway analysis identified the most significant pathways in the dataset.

There were previous reports on expression in plasma or serum miRNA profile of glioma (20,27). We found 20 miRNAs either up- or down regulated which were significant different. We would have preferred to do a complete miRNA profiling by RT-qPCR in all the 139 subjects, but due to high costs this was not possible. We only analyzed significantly different 20 miRNAs in the main study. An additional factor was that inclusion of more than 100 miRNAs would have made it very much difficult to evaluate whether an observed change was truly significant or simply due to chance because of a large number of miRNA analyzed. Instead we selected the most promising 20 miRNAs that had shown a significant expression change. Of these 20 miRNAs, only miR-576-5p, miR-340, miR-626, miR-320, let-7g-5p and miR-7-5P were included in this GO analysis and pathway analysis. Furthermore, in a study on glioma where RNA had been extracted and purified from whole blood, showed different expression. Normal biological variation of miRNA levels in plasma together with variation introduced during sample treatments (from blood sampling to RT-qPCR) may affect the observed expression of miRNAs (35). Also in the initial report on miRNA in serum, most of the miRNAs were detected in both serum and blood cells. However, only a small number of miRNAs were uniquely present in either serum or blood cells, indicating that most serum miRNAs may be derived from circulating blood cells (28,36). In addition, the miRCURY LNA RT-qPCR system used for detection of known plasma miRNAs has been found to be more sensitive and reproducible than the initial microarray systems and show even more sensitivity and linearity than TaqMan methods at low concentration of plasma miRNA (37,38).

Previous studies on identifying serum miRNA-based biomarkers generally focused on individual miRNA. However, the specificity of biomarkers based on a single miRNA is generally questionable and poor (27,28). In this study, it showed that the combination of six significantly diverse serum miRNAs would be a more comprehensive indicator for the diagnosis of GBM, however, it has not yet been precisely investigated in the tissues or serum of glioma patient. There are several limitations in our study. Firstly, the sample size is small. Whether this six-member serum miRNA profile can be established as a routine biomarker for pertussis diagnosis will require more investigation. Secondly, the testing of a large number of clinical samples will be required to compare and confirm the results of this study. Finally, we only evaluated partially the dysregulated miRNAs, thus some miRNAs other than those reported herein might also be identified as serum biomarkers for GBM diagnosis in future studies.

The secretory mechanism and biological function, as well as the meaning of the existence of extracellular miRNAs, remain largely unclear. Because of the lack of any biomarker in peripheral blood that is in clinical use for glioblastoma, the findings of our study may provide the scientific basis for further studies using miRNAs for the follow-up of patients diagnosed with glioblastoma. Considering the biological relevance of miRNAs to GBM and recent studies of circulating miRNAs in serum by others, we hypothesize that the serum miRNAs may serve as novel biomarkers for the diagnosis and evaluation of GBM.

Collectively, we have demonstrated that a unique six-member serum miRNA expression profile could serve as a non-invasive biomarker for GBM diagnosis.

Acknowledgements

The authors thank Shizhu Yu, Department of Neuropathology, Neurology Institute, Tianjin Medical University General Hospital, Tianjin Neurological Institute, China, for his technical assistance.

References

1. Tzadok S, Beery E, Israeli M, *et al*: *In vitro* novel combinations of psychotropics and anti-cancer modalities in U87 human glioblastoma cells. Int J Oncol 37: 1043-1051, 2010.
2. Han L, Zhang K, Shi Z, *et al*: LncRNA profile of glioblastoma reveals the potential role of lncRNAs in contributing to glioblastoma pathogenesis. Int J Oncol 40: 2004-2012, 2012.
3. Wang Y, Chen L, Bao Z, *et al*: Inhibition of STAT3 reverses alkylator resistance through modulation of the AKT and β-catenin signaling pathways. Oncol Rep 5: 1173-1180, 2011.
4. Wang LF, Fokas E, Bieker M, *et al*: An HX Increased expression of EphA2 correlates with adverse outcome in primary and recurrent glioblastoma multiforme patients. Oncol Rep 19: 151-156, 2008.
5. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 23: 281-297, 2004.
6. Ambros V: The functions of animal microRNAs. Nature 16: 350-355, 2004.
7. Grimson A, Farh KK, Johnston WK, *et al*: MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27: 91-105, 2007.
8. He L and Hannon GJ: MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5: 522-231, 2004.
9. Kim VN: MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6: 376-385, 2005.
10. Zhao C, Tian F, Yu Y, *et al*: miRNA-dysregulation associated with tenderness variation induced by acute stress in Angus cattle. J Anim Sci Biotechnol 3: 12-17, 2012.
11. Cuellar TL and McManus MT: MicroRNAs and endocrine biology. J Endocrinol 187: 327-332, 2005.
12. Poy MN, Eliasson L, Krutzfeldt J, *et al*: A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432: 226-230, 2004.
13. Cheng H, Zhang L, Cogdell DE, *et al*: Circulating plasma MiR-141 is a novel biomarker for metastatic colon cancer and predicts poor prognosis. PLoS One 6: e17745, 2011.
14. Ng EK, Chong WW, Jin H, *et al*: Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut 58: 1375-1381, 2009.
15. Rabinowits G, Gercel-Taylor C, Day JM, *et al*: Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer 1: 42-46, 2009.
16. Resnick KE, Alder H, Hagan JP, *et al*: The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. Gynecol Oncol 112: 55-59, 2009.
17. Taylor DD and Gercel-Taylor C: MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110: 13-21, 2008.
18. Yaman Agaoglu F, Kovancilar M, Dizdar Y, *et al*: Investigation of miR-21, miR-141, and miR-221 in blood circulation of patients with prostate cancer. Tumour Biol 32: 583-588, 2011.
19. Li W1, Yuan Y, Huang L, *et al*: Metformin alters the expression profiles of microRNAs in human pancreatic cancer cells. Diabetes Res Clin Pract 96: 187-195, 2012.
20. Roth P, Wischhusen J, Happold C, *et al*: A specific miRNA signature in the peripheral blood of glioblastoma patients. J Neurochem 118: 449-457, 2011.
21. Kapranov P, Cheng J, Dike S, *et al*: RNA map reveals new RNA classes and a possible function for persvasive transcription. Science 316: 1484-1488, 2007.
22. Taft RJ, Pang KC, Mercer TR, *et al*: Non-coding RNAs: regulators of disease. Pathology 220: 126-139, 2010.
23. Zhang W, Edwards A, Fan W, *et al*: miRNA-mRNA correlation-network modules in human prostate cancer and the differences between primary and metastatic tumor subtypes. PLoS One 7: e40130, 2012.
24. Williams AE: Functional aspects of animal microRNAs. Cell Mol Life Sci 65: 545-562, 2008.
25. Place RF, Li LC, Pookot D, *et al*: MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci USA 105: 1608-1613, 2008.
26. Brennecke J, Hipfner DR, Stark A, *et al*: Bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. Cell 113: 25-36, 2003.
27. Chen X, Ba Y, Ma L, *et al*: Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18: 997-1006, 2008.
28. Kosaka N, Iguchi H and Ochiya T: Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 101: 2087-2092, 2010.
29. Li Z, Gu X, Fang Y, *et al*: microRNA expression profiles in human colorectal cancers with brain metastases. Oncol Lett 3: 346-350, 2012.
30. Díaz-Prado S, Cicione C, Muiños-López E, *et al*: Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes. BMC Musculoskelet Disord 13: 144, 2012.
31. Giles KM, Brown RA, Epis MR, *et al*: miRNA-7-5p inhibits melanoma cell migration and invasion. Biochem Biophys Res Commun 430: 706-710, 2013.
32. Dong L, Han C, Zhang H, *et al*: Construction of a recombinant lentivirus containing human microRNA-7-3 and its inhibitory effects on glioma proliferation. Neural Regener Res 7: 2144-2150, 2012.
33. Bader AG, Brown D and Winkler M: The promise of microRNA replacement therapy. Cancer Res 70: 7027-7030, 2010.
34. Giles KM, Brown RA, Epis MR, *et al*: miRNA-7-5p inhibits melanoma cell migration and invasion. Biochem Biophys Res Commun 430: 706-710, 2013.
35. McDonald JS, Milosevic D, Reddi HV, *et al*: Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem 57: 833-840, 2011.
36. Jorde R, Svartberg J, Joakimsen RM and Coucheron DH: Plasma profile of microRNA after supplementation with high doses of vitamin D3 for 12 months. BMC Res Notes 5: 245, 2012.
37. Chen Y, Gelfond JA, McManus LM and Shireman PK: Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genomics 10: 407, 2009.
38. Jensen SG, Lamy P, Rasmussen MH, *et al*: Evaluation of two commercial global miRNA expression profiling platforms for detection of less abundant miRNAs. BMC Genomics 12: 435, 2011.