Supplementary Data

Circulating miR-16-5p and miR-19b-3p as Two Novel Potential Biomarkers to Indicate Progression of Gastric Cancer

Jingpu Zhang, Yang Song, Chunlei Zhang, Xiao Zhi, Hualin Fu, Yue Ma, Yunsheng Chen, Fei Pan, Kan Wang, Jian Ni, Weilin Jin, Xianli He, Haichuan Su, Daxiang Cui

Screening miRNAs by Microarray

Each slide contained eight individual microarrays, each with 60,000 features. Total RNA (100ng) derived from plasma samples were directly end-labeled with Cy3. The labeling and hybridization were performed according to the manufacture's protocol (Agilent Technologies, Santa Clara, CA). Slides were scanned on an Agilent microarray scanner (model G2565A) at 100% and 5% sensitivity settings.

The microarray image information was converted into spot intensity values using Feature Extraction Software Rev. 9.5.3 (Agilent Technologies, Santa Clara, CA). The signal after background subtraction was exported directly into the GeneSpring GX10 software (Agilent Technologies, Santa Clara, CA) for quartile normalization and further data analysis. The significance of plasma miRNA levels between GC and N groups was determined by one-way analysis of variance (ANOVA) with Benjamini-Hochberg correction (P<0.01 or P<0.05), or t-test for unpaired, unequal variance (Welch) with Benjamini-Hochberg correction (P<0.01 or P<0.05). Hierarchical clustering with Pearson Correlation was employed in differential expressed miRNAs between GC and N groups discovered by microarray analysis. And the two-way Venn diagram was applied to identify the number of identical differential expressed miRNAs from the two batches of microarray profilings.

Plasma preparation and RNA isolation

2-4mL of peripheral blood was drawn into EDTA-treated tubes. Within 1 hour of

collection at room temperature, the tubes were subjected to centrifuge for 10 min (820 *g*, 4°C). And the upper-layer plasma was transferred into fresh RNase/DNase-free 1.5mL microfuge tubes followed by further centrifugation for 10 min (16,000 *g* , 4°C). Subsequently, the 1-2mL of supernatant was transferred to another fresh tube and stored at –80 °C.

Total RNA was isolated from 200μL plasma using mirVana Paris Kit (SKU: AM1556, Ambion, Texas, USA). Following the manufacturer's protocol for liquid samples, some modifications were made: Firstly, to allow for normalization of sample-to-sample variation in the RNA isolation step, synthetic C. elegans miRNA cel-miR-39 (0.5μM, 2.5μL) was added to each denatured sample after combining the plasma sample with Denaturing Solution [1]; Secondly, samples were extracted twice with an equal volume of acid-phenol chloroform. RNA was eluted with 100μL pre-heated (95°C) nuclease-free water. The concentration was quantified by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Delaware, USA). The concentration of the total RNA extracted from plasma ranged from 2.1 to 10.2ng·μL^{-1}. The extracted total RNA was stored at –80 °C for further use.

qRT-PCR assay of screened miRNAs

The quantification of miRNA was performed using TaqMan MicroRNA Reverse Transcription Kit (SKU: 4366596, Applied Biosystems, California, USA) to prepare the reverse transcription (RT) master mix. And TaqMan microRNA assay (SKU: 4427975, Applied Biosystems, California, USA) was served to provide the gene-specific stem-loop primers, gene-specific primers and TaqMan probers respectively for the RT reaction and the following qPCR reaction. In addition, the GoTaq Probe qPCR Master Mix (2×, P/N: A6101, Promega, Wisconsin, USA) was used. The RT master mix was prepared in aliquots of 77.0μL that contained 1.7μL 100mM dNTPs, 11μL Multiscribe Reverse-Transcriptase (50U·μL^{-1}), 16.5μL 10× Reverse-transcription Buffer, 2.1μL RNase-Inhibitor (20U·μL^{-1}) and 45.7μL nuclease-free water in each aliquot. And TaqMan microRNA assay used in the study were listed in Table S1.

For all the 8 microRNAs selected for qRT-PCR, the RT reaction was carried out in

10μL system containing 4.66μL RT mix prepared above, 3.34μL RNA extract and 2μL RT gene-specific primer. To synthesize cDNA, the resulting reaction mixtures were incubated at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and then held at 4°C. Then, 3μL cDNA was kept from the RT reaction for further use, and 2.5μL nuclease-free water was added into the left cDNA mixtures for qPCR. The following qPCR reaction that contained 10.5μL GoTaq Probe qPCR Master Mix, 1.05μL gene-specific primer/probe and 9.45μL cDNA dilution solution was run on BioRad-iQ5 and the reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. Each reaction was carried out in duplicates except for miR-671-5p, which was carried out in triplicates in a different qPCR reaction mixtures containing 15.56μL GoTaq Probe qPCR Master Mix, 1.56μL gene-specific primer/probe and 14μL cDNA solution diluted by adding 4μL nuclease-free water into the 10μL cDNA from RT reaction.

The assigning baseline and threshold for Ct determination was automatically set. MiRNAs with a Cq value equal to or below 35 and the detection rate reaching up to 75% were selected for the following detection (Fig.1) [2, 3]. The targeted miRNAs and the corresponding reference miRNAs were properly arranged in the strip tubes to avoid bias related to geographic location on the PCR plate (96-well). No template control (NTC) was included for each assay on the plate.

The concentration of spiked-in cel-miR-39

To determine the proper concentration of spiked-in cel-miR-39, we added different concentrations of cel-miR-39 to the same plasma sample, and qRT-PCR was subsequently conducted. All procedures were carried out the same as the protocol in plasma preparation and RNA isolation except the following procedures: Firstly, one plasma sample was randomly selected and allocated into four aliquots, each with 200μL. Secondly, a set of cel-miR-39s at gradient concentrations of 0.005, 0.05, 0.5, 5μM in 2.5μL solution was separately added into the denatured plasma mixtures after combining the plasma samples with Denaturing Solution. The concentration of RNA extracted from the spiked-in plasma ranged from 2.7 to 3.7ng·μL^{-1}.

TaqMan qRT-PCR was conducted to measure the spiked-in miRNA. The RT step

was performed as described in qRT-PCR assay of screened miRNAs. As for qPCR, an independent reaction system was employed, which contain 16.5μL GoTaq Probe qPCR Master Mix, 1.65μL gene-specific primer/probe and 14.85μL cDNA dilution solution by adding 57.8μL nuclease-free water to 10μL cDNA solution from the RT step. Each miRNA was amplified in triplicates. The rest procedures were operated the same as before.

The amplification curves and the corresponding Ct values of the spiked-in cel-miR-39 at gradient concentrations were shown in Fig. S1. From the amplification curves, we can see that with the increase on the concentration of cel-miR-39, the amplification curves moved stepwise from the right side to the left side. Correspondingly, the Ct values decreased from 31.96 to 13.51. And when the concentration of cel-miR-39 was 0.5μM, the Ct value lay in 20.97, which was available for data normalization, and thus 0.5μM of cel-miR-39 in 2.5μL volume was adopted to add into 200μL plasma.

The comparisons of expression levels of miR-16-5p and miR-19b-3p by age and sex

Considering the significant difference of age between GC and N cohorts in the study (Table 1), the expression levels of miR-16-5p and miR-19b-3p were compared separately in 155 GC patients and 111 N controls in the training and validation phases by age as well as sex to examine if the difference would adversely affect the biomarkers' screening. As depicted in Fig. S4, the cohort with age$\geqslant$50 was allocated together into a group and the remainder into the other one, and disappointedly there was no age information for one normal subject. It is clear that both miR-16 and miR-19b was expressed at the same level with no significant difference between the different age groups, and the same case for the sex groups. Therefore, we may conclude that there existed little effect of the age and sex difference on the biomarkers' screening, which was also proved in Nicolai and other's studies [4, 5].

Table S1. TaqMan microRNA assays for qRT-PCR in the training phase.

Systematic Name	MiRBase accession No.	Assay ID
cel-miR-39	MIMAT0000010	200
hsa-miR-16	MIMAT 0000069	391
hsa-miR-101-3p	MIMAT 0000099	2253
hsa-miR-19b	MIMAT 0000074	396
hsa-miR-671-5p	MIMAT 0003880	197646_mat
hsa-miR-21-5p	MIMAT 0000076	397
hsa-miR-144-3p	MIMAT 0000436	2676
hsa-miR-15a-5p	MIMAT 0000068	389
hsa-miR-3940-5p	MIMAT 0019229	462391_mat
hsa-miR-4298	MIMAT 0016852	465290_mat

Table S2. Differentially expressed miRNAs (*P*<0.05) collectively selected from two sets of microarray profiling.

Name	Accession	Regulation	BH13053		BH13333	
			P value	FC	*P* value	FC
hsa-miR-19b-3p	MIMAT0000074	down	0.00329	0.439	0.03310	0.240
hsa-miR-3162-5p	MIMAT0015036	up	0.01935	1.853	0.01202	1.444
hsa-miR-4466	MIMAT0018993	up	0.00168	2.111	0.02136	1.183
hsa-miR-4532	MIMAT0019071	up	0.00119	1.744	0.03375	1.522
hsa-miR-671-5p	MIMAT0003880	up	0.02281	1.285	0.00831	1.931

Table S3. Differentially expressed miRNAs (*P*<0.01, FC<0.5 or >2) respectively selected from two sets of microarray profiling.

Name	Accession	Regulation	*P* value	FC
BH13053(14 miRNAs)				
hsa-let-7a-5p	MIMAT0000062	down	0.00011	0.083
hsa-miR-101-3p	MIMAT0000099	down	0.00043	0.380
hsa-miR-106b-5p	MIMAT0000680	down	0.00006	0.399
hsa-miR-144-3p	MIMAT0000436	down	0.00149	0.342
hsa-miR-15a-5p	MIMAT0000068	down	0.00189	0.276
hsa-miR-16-5p	MIMAT0000069	down	0.00103	0.232
hsa-miR-18b-5p	MIMAT0001412	down	0.00045	0.189
hsa-miR-19b-3p	MIMAT0000074	down	0.00330	0.439
hsa-miR-301a-3p	MIMAT0000688	down	0.00034	0.112
hsa-miR-363-3p	MIMAT0000707	down	0.00958	0.224
hsa-miR-3940-5p	MIMAT0019229	up	0.00498	2.090
hsa-miR-4298	MIMAT0016852	up	0.00358	2.313
hsa-miR-4466	MIMAT0018993	up	0.00168	2.111
hsa-miR-4745-5p	MIMAT0019878	up	0.00048	8.332
BH13333(7 miRNAs)				
hsa-miR-4516	MIMAT0019053	down	0.00429	0.406
hsa-miR-451a	MIMAT0001631	down	0.00857	0.066
hsa-miR-5196-5p	MIMAT0021128	down	0.00264	0.279
hsa-miR-6723-5p	MIMAT0025855	down	0.00085	0.188
hsa-miR-1304-3p	MIMAT0022720	up	0.00150	4.090
hsa-miR-6508-5p	MIMAT0025472	up	0.00303	3.711
hsa-miR-6515-5p	MIMAT0025486	up	0.00384	2.984

Table S4. The expression levels of miR-19b-3p normalized to two reference miRNAs in the total training and validation phases.

	aveΔCt	Sd	$2^{-ave\Delta\Delta Ct}$	*P* value
T1	8.525	0.872	0.715	0.021
T2	8.763	1.115	0.607	0.001
T3	9.314	1.469	0.414	0.000
T4	8.988	0.763	0.519	0.000
G1	8.568	0.996	0.694	0.126
G2	8.782	0.740	0.599	0.000
G3	9.196	1.436	0.449	0.000
GC	8.915	1.256	0.546	0.000
N	8.042	0.983	1	

The items were stated as in Table 3. Mann-Whitney U test was used to determine statistical significance at the level of $P < 0.05$.

Table S5. Performance of miR-19b-3p normalized to two reference miRNAs in the differential diagnosis of GC cases with different TNM stages and differentiation grades from N controls in the total training and validation phases.

	AUC (95%CI)	Cutoff	Sensitivity	Specificity
T1	0.633 (0.527,0.739)	8.309	0.667	0.586
T*	0.660 (0.554,0.766)	8.921	0.545	0.758
T2	0.702 (0.589,0.815)	8.030	0.885	0.505
T3	0.811 (0.746,0.875)	8.689	0.712	0.739
T4	0.776 (0.682,0.871)	8.140	0.880	0.541
G1	0.616 (0.489,0.742)	7.818	0.882	0.414
G2	0.710 (0.627,0.792)	8.115	0.776	0.541
G3	0.788 (0.721,0.856)	8.098	0.906	0.532
GC	0.734 (0.674,0.794)	8.115	0.819	0.541

*, ROC curve conducted between GC cases with TNM Ⅰ stage and those with the others; T1-4 and G1-3, as stated in Table 3.

Table S6. The expression levels of miR-16-5p and miR-19b-3p in the supplementary phase Ⅱ

	aveΔCt	Sd	$2^{-ave\triangle\triangle Ct}$	*P* value
h16c39				
post-operation	8.825	0.422	1.269	0.839
pre-operation	9.169	1.617	-	-
h19bc39				
post-operation	13.994	1.121	0.630	0.037
pre-operation	13.328	1.027	-	-
h19bavec39h16				
post-operation	9.581	0.967	0.560	0.020
pre-operation	8.743	1.391	-	-

aveΔCt, the mean value of the normalized Ct values of the miRNA for the special cohort; Sd, the corresponding standard deviation to aveΔCt; $2^{-ave\triangle\triangle Ct}$, the ratio of the average expression level of the miRNA for the pre-operative GC cohort to that for the post-operative one. h16c39, h19bc39 and h19bavec39h16 were stated as in Fig. 3. Mann-Whitney U test was used to determine statistical significance at the level of $P < 0.05$.

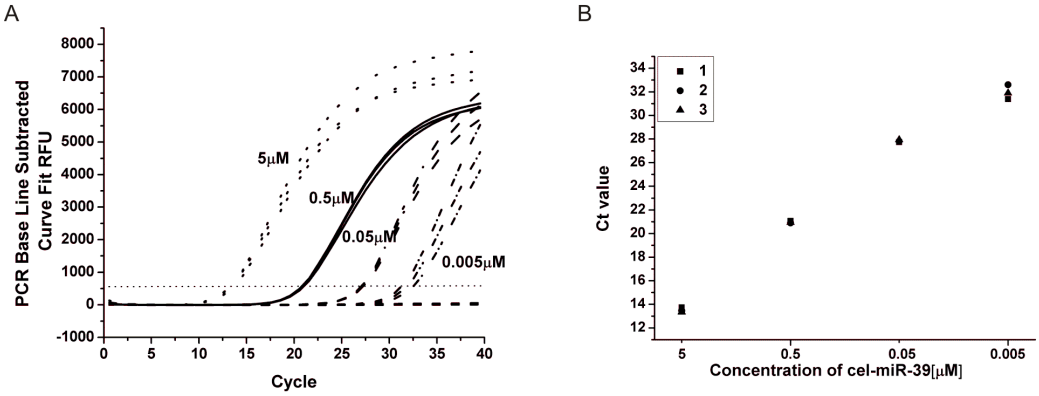

Figure S1. Amplification curves (**A**) and the corresponding Ct values (**B**) of the spiked-in cel-miR-39 at gradient concentrations.

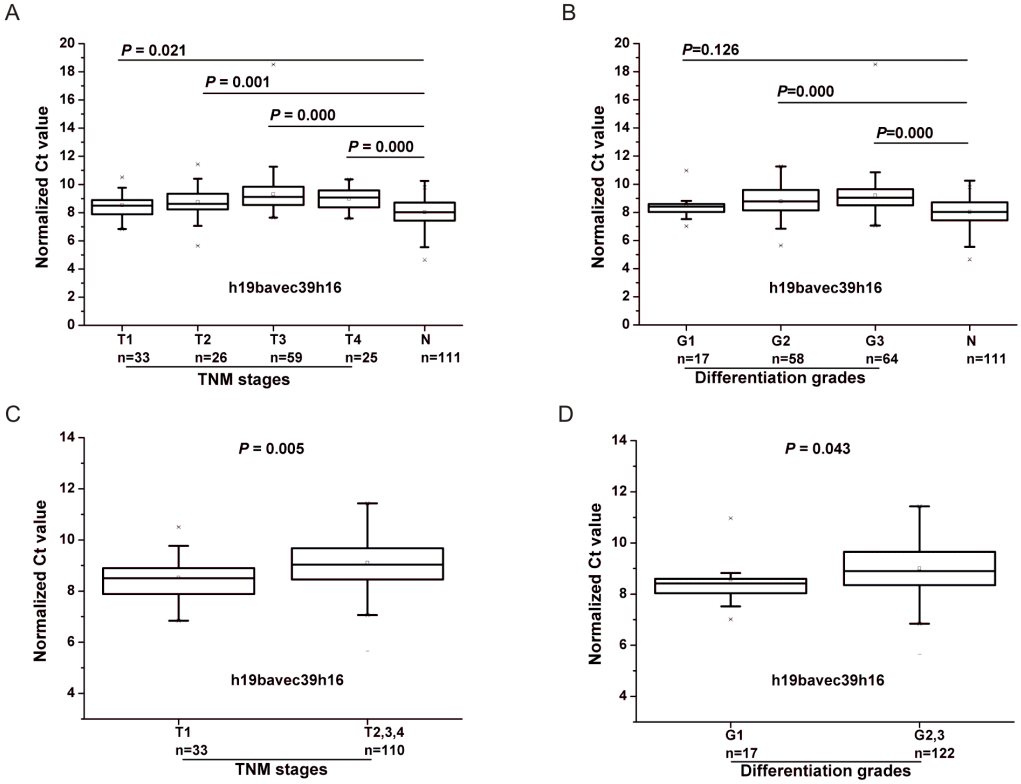

Figure S2. Differential expression levels of miR-19b-3p normalized to two reference miRNAs in the total training and validation phases. (**A**)Boxcharts respectively between GC cases with different TNM stages and N controls. (**B**)Boxcharts respectively between GC cases with different differentiation grades and N controls. (**C**) Boxcharts between GC cases with T1 stage and those with the others. (**D**) Boxcharts between GC cases in G1 and those in the others. T1-4 and G1-3, as stated in Table 3; h16c39 and h19bc39, as stated in Fig. 3. Mann-Whitney U test was used to determine the statistical significance at the level of $P<0.05$.

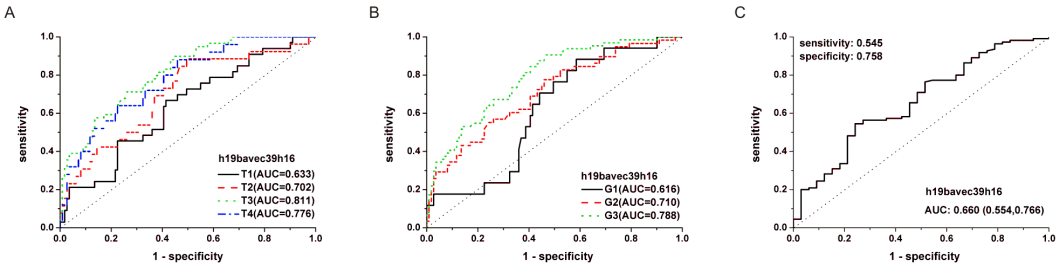

Figure S3. ROC curves for miR-19b-3p normalized to two reference miRNAs in the total training and validation phases. (**A**) Curves to distinguish GC cases with different TNM stages from N controls; (**B**) Curves to distinguish GC cases with different differentiation grades from N controls (**C**) Curve to distinguish GC cases with T1 stage from those with the others. T1-4 and G1-3, as stated in Table 3; h19bavec39h16, as stated in Fig. 3.

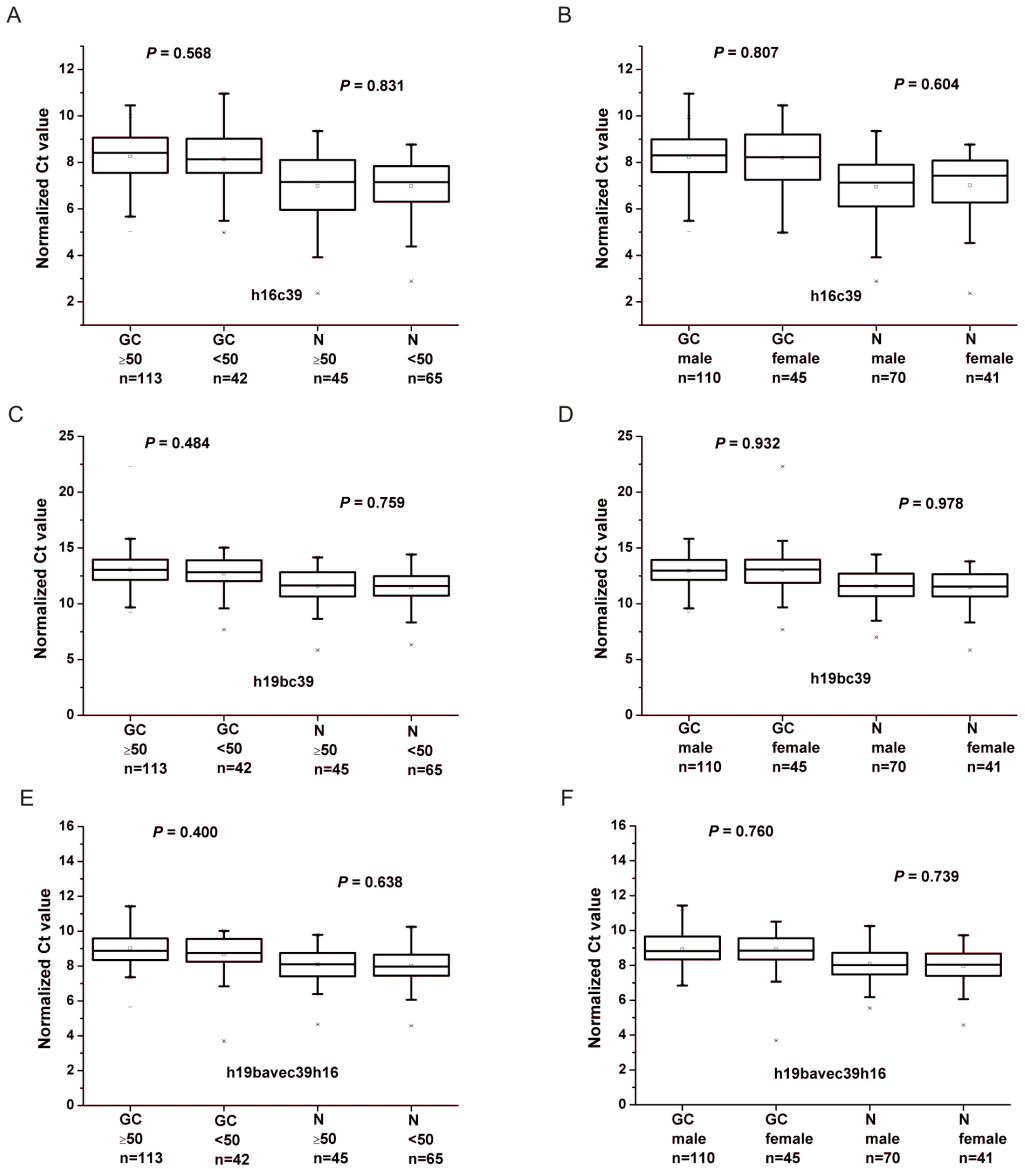

Figure S4. The expression levels of miR-16-5p (**A,B**), miR-19b-3p (**C,D**) normalized to the exogenous miRNA, and miR-19b-3p normalized to two reference miRNAs (**E,F**) in the total training and validation phases allocated by age(**A,C,E**) and sex(**B,D,F**). h16c39, h19bc39 and

h19bavec39h16 were stated as in Fig. 3. Mann-Whitney U test was used to determine statistical significance at the level of $P<0.05$.

Reference

1. Mitchell. Circulating microRNAs as stable blood-based markers for cancer detection. P Natl Acad Sci USA. 2008; 105: 10513.
2. Mestdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol. 2009; 10: 9.
3. Liu R, Zhang CN, Hu ZB, et al. A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. Eur J Cancer. 2011; 47: 784-91.
4. Schultz NA, Dehlendorff C, Jensen BV, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. J Am Med Assoc. 2014; 311: 392-404.
5. Reid G, Kirschner MB, van Zandwijk N. Circulating microRNAs: association with disease and potential use as biomarkers. Crit Rev Oncol Hemat. 2011; 80: 193-208.