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Original Article

Analysis of plasma MicroRNAs to identifying early diagnostic molecule for gastric cancer

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Abstract: Gastric cancer (GC) remains the second leading cause of cancer-related death worldwide. Owing to the lack of early diagnostic techniques, GC is often diagnosed at advanced stage and that leading to low survival rate. Growing evidences have been suggesting that circulating microRNAs play an important role in earlier diagnostic of disease. In the present study, we analyze the circulating miRNAs expression in plasma of volunteers with/without GC aiming to identifying early diagnostic biomarkers. Plasma samples were collected form 6 volunteers including 3 early patients with GC and 3 healthy adults. And then miRNAs microarrays were performed to detect the expression profile of miRNAs in these plasma samples. For further validate the results from miRNAs microarray, qRT-PCR was performed. Finally, target genes of miRNAs were predicted by bioinformatic means. Compared to control plasma, 11 up-regulated and 13 down-regulated miRNAs were detected in the plasma from earlier patients with GC (fold change ≥ 2, $P < 0.05$). Then, 5 differential expression miRNAs (miR-223, miR-19b-2*, miR-194*, miR-141, miR-1233) were chose to confirm by qRT-PCR. The result is nearly consistent with previous data from miRNAs microarray. Finally, 53 target genes of the 5 miRNAs are predicted by bioinformatics. These differential expression miRNAs may be used as biomarker candidates for minimally invasive diagnosis of early patients with GC in the future.

Keywords: Gastric cancer, miRNAs, early diagnosis, biomarkers, plasma

Introduction

Gastric cancer (GC) remains the second leading causes of cancer-related death worldwide. Although diagnostic technique and treatment methods have been improving over the past few decades, the prognosis of GC is still poor. Meanwhile, most of patients are diagnosed at the advanced stage of GC so that missing the best opportunity of treatment [1]. Biomarkers have been hoping to be promising targets for improvement early diagnosis rate. Circulating miRNAs as early diagnostic molecule own minimally invasive characteristic compared to biopsy [2].

miRNAs are highly conserved small non-coding RNA molecules (17~22 nt), which regulate their target genes usually on the post-transcriptional level by binding to complementary sequences on target mRNA transcripts (mRNAs) [3]. They actively participate in the modulation of important cell physiological processes, and they are involved in the pathogenesis of many diseases, including cancer [2]. A better understanding of the role that miRNAs play in these diseases, especially in gastric cancer, could lead to the development of new diagnostic and therapeutic tools.

Since the first description of miRNAs in 1993 [4], nearly 2,000 human miRNA genes have known, and more than 1,000 new ones are continuously discovered [5]. In recent years, miRNAs either directly through binding to the target mRNA or indirectly through repressing nonsense-mediated RNA decay [6, 7]. MicroRNAs are not only playing an essential role in cellular processes, but also contributing to many human pathologies including cancer [8]. Subsequently, a multitude of studies about miRNA expression changes in cancer have been reported.

Table 1. Primers for real time PCR in this study

Primers	Sequence (5'-3')
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
miR-223-F	GCTGTCAGTTTGTCAAATAC
miR-223-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGT
miR-19b-2*-F	AGTTTTGCAGGTTTGCAT
miR-19b-2*-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGAAAT
miR-194*-F	CCAGTGGGGCTGCTGTTA
miR-194*-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGATA
miR-141-F	GCTAACACTGTCTGGTAAAG
miR-141-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCATCT
miR-1233-F	TGAGCCCTGTCCTCCC
miR-1233-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGCGG
Universal primer R	GTGCAGGGTCCGAGGT

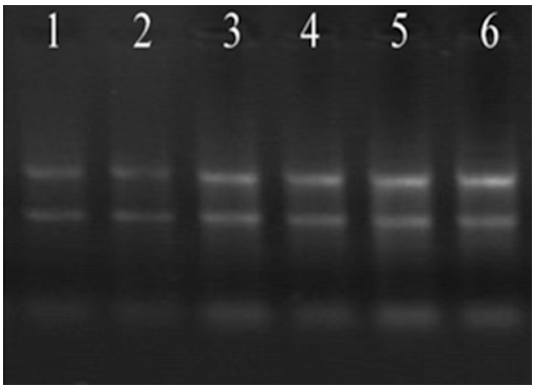

Figure 1. Agarose gel electrophoresis of denatured RNA. 1-3: Control groups 4-6: Gastric cancer.

In this article, we investigated the change of differentially expressed miRNAs in plasma of gastric cancer patients through microarray analysis and predicted their target genes with prediction software aiming to identifying novel early diagnostic molecules for GC

Materials and methods

Plasma samples

Three plasma samples of pathologically diagnosed early gastric cancer patients and 3 normal plasma samples as controls were collected from gastric cancer patients of the First Affiliated Hospital, Anhui Medical University, with informed consent to the study. Pathological diagnosis and classification of all patients were conducted by experienced pathologists according to the 2003 WHO Tumor Classification Standard. Pathological diagnoses of the included patients are shown in Supplementary materials. Informed consent from all patients was obtained and this study was approved by Ethics Committee of the First Affiliated Hospital, Anhui Medical University.

RNA isolation

Peripheral blood samples were collected, mixed and centrifuged in the morning. Total RNA was isolated utilizing mirVana™ PARIS™ Kit (Applied Biosystem, USA), and then swabbed off it and concentrated in vacuum. RNA titer was conducted using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA concentration and OD (A260/A280) were recorded. Then RNA quantification was conducted by electrophoresis.

MiRNA microarray analysis

After the samples' dephosphorylation and denaturation, RNA was marked by marker mixture (10 × T4 RNA Ligase Buffer, Cyanine 3-pCp, T4 RNA Ligase). 10 × GE blockers (Agilent, USA) were used to prepare hybrid samples, then, hybridization was conducted utilizing hybrid box (Agilent, USA). After washing the chips, we conducted microarrays scanning by Laser Scanner (Agilent, USA) and Scan Control Software (Agilent, USA). Data analysis was conducted using GenesPring GX 10.0.

Fluorescent quantitative RT-PCR validation

Primer sequences were designed as **Table 1**. We designed RT primer with stem-loop taking sequence 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC-3' as a reference, using Primer Premier 5.0 to design forward primer according to cDNA sequences after reverse transcription. At last we added GC at 5' of PCR forward primer based on Tm calculated by Primer Premier 5.0 to make the temperature of forward primer to be around 60°C. The reverse primer was a general sequence

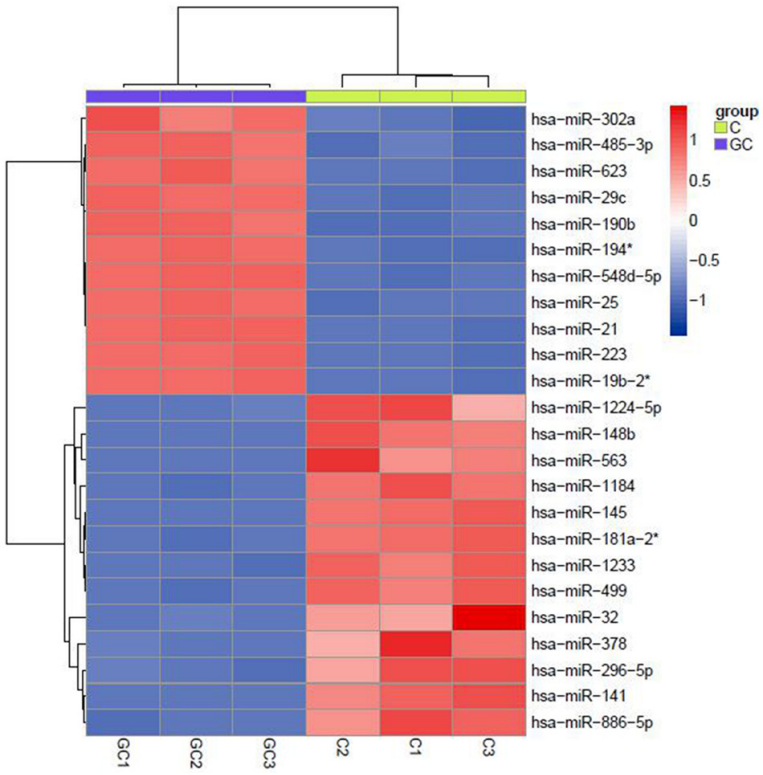

Figure 2. miRNA microarray scanning of plasma samples from gastric cancer patients and normal adults.

5'-GTGCAGGGTCCGAGGT-3'. U6 RNAof snRNA was as internal standard of normalization. Then primers were used to synthesize cDNA which would be samples of Real TimePCR.

Target gene prediction of miRNAs

Targetscans (http://www.targetscan.org/), Pictar (http://pictar.mdc-berlin.de/) and Miranda (http://www.microrna.org/microrna/home.do) were used to analyze miRNA target genes.

Statistical analysis

GenesPring Gx10.0 Software was used to conduct bioinformatic analysis of the data extracted by Feature Extraction Software. After introducing data, percentile shift was used for normalization and logarithmic transformation. Unexpressed probes were filtered out by parameter Flag Value; t-test was used to analysis differentially expressed miRNAs in tumor samples and adjacent normal tissues of cancer; fold change test was used to screen miRNAs whose expression differentiation was more than 2 times. Data analysis was conducted using SPSS 13.0 software, t-test. $P < 0.05$ was considered statistically significant.

Results

Total RNA concentration and quality

OD260/OD280 ratio ranged among 1.8~2.0 which indicated a high purity of total RNA and it was free from DNA and protein contamination. The agarose gel electrophoresis showed that the density of rRNA 28S band was about 2-fold of 18S band (**Figure 1**), which demonstrated a good integrity of the extracted total RNA. Quality testing showed that the total RNA samples met the quality requirements.

Expression differences of miRNAs in plasma between gastric cancer patients and healthy human

The results of miRNA microarray showed that 24 differentially expressed of miRNAs were found compared to control groups, among which 11 miRNAs were up-regulated and 13 down-regulated (fold change ≥ 2, *p*-value < 0.05). The differentially expressed miRNAs are shown in **Figure 2**, with fold change value and adjusted *P*-value. These results indicate that these miRNAs may be involved in the development and progress of GC, and can be used as early diagnostic molecule for GC.

Fluorescent quantitative RT-PCR validation

Because of the indirect association between miRNA microarrays and miRNA expression characteristics, disadvantages like specificity and low sensitivity still exist, which may lead to a false positive result. A further fluorescent quantitative RT-PCR experiment was conducted for more accurate results.

Five miRNAs, either over 6 times up-regulated ones (hsa-miR-223, hsa-miR-19b-2* and hsa-miR-194*) or more than 3 times down-regulated ones (hsa-miR-141 and hsa-miR-1233),

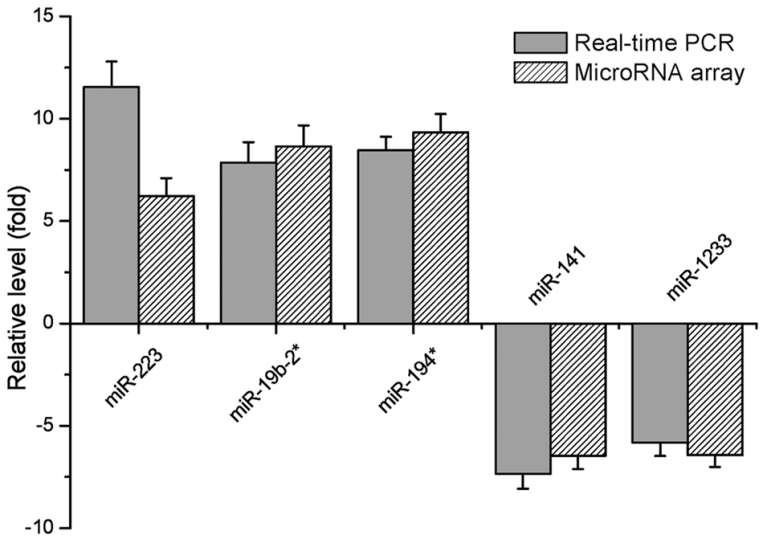

Figure 3. Relative level of differentially expressed miRNAs based on the results of real-time PCR and microRNA array.

were the materials of quantitative RT-PCR validation. PCR result showed an up-regulated expression of miR-223, miR-19b-2*, miR-194* in gastric cancer patients' plasma, and a down-regulated expression of miR-141 and miR-1233 (**Figure 3**), which demonstrated a consistent result with miRNA microarrays.

Target gene prediction of differentially expressed miRNAs

For further understand the function of the five miRNAs mentioned above, their target genes were predicted by bioinformatics. As shown in **Table 2**, each miRNA at least match to 5 target genes. Functional analysis shown that these genes are involved in many important life processes such as metastasis.

Discussion

Presently, a large number of studies discovered that more and more miRNAs are involved in tumorigenesis of gastric cancer and playing crucial roles in its evolution, invasion, metastasis and angiogenesis, etc [9-11]. With deeper and deeper understanding the regulating effects of miRNAs on genetic expression and miRNA differential expression, the potential value of miRNAs in tumor diagnosis, therapy and prognosis evaluation was gradually realized [12, 13].

In our present research, differential expression profile of miRNAs in plasma of gastric cancer patients was obtained, which provides useful data for further studying the regulating effects of miRNAs on molecular mechanism of the tumorigenesis and progress of gastric cancer. We found 11 miRNAs were found up-regulated in the plasma of gastric cancer patients, 13 were down-regulated, in which, the expressions of 5 miRNAs were significantly up-regulated (hsa-miR-223, hsa-miR-19b-2* and hsa-miR-194*) or down-regulated (hsa-miR-141 and hsa-miR-1233).

The plasma level of hsa-miR-223 was significantly higher in gastric cancer patients than in healthy controls, promoting gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3 [14], and plasma hsa-miR-223 is a novel potential biomarker for gastric cancer detection as well [15]. Hsa-miR-19b-2* were not detected in osteosarcoma [16] and T cell leukemia [17], but it was found significantly raised in this study, suggesting that hsa-miR-19b-2* might specifically or more highly expressed in gastric cancer. Contrary to our results, it was found by other researchers that hsa-miR-194* and hsa-miR-1233 were totally altered in an opposite way in gastric cancers or other ones: hsa-miR-194* was significantly down-regulated in intestinal-type gastric cancers [18], and hsa-miR-1233 level is distinctly increased in renal cell carcinoma [19]. Consistent with our results, hsa-miR-141 was significantly down-regulated in gastric cancer tissues and serum of ovarian cancer patients [20, 21]. These facts demonstrated that the above significantly altered miRNAs in serum may serve as potential biomarkers or prognosis for gastric cancer [15, 19, 21-23].

Based on the analysis of online prediction, differentially expressed miRNAs were shown to take parts in regulating 74 predicted target genes, and they probably function in many physiological processes. With further analysis, we found there are no genes that are regulated

Table 2. Target genes predicted with Targetscan, Pictar and Miranda softwares

miRNA	Fold Change	Target Genes
hsa-miR-223	6.22568749	ARL6IP2, C13ORF18, FBXO8, HHEX, INPP5B, NFIA, PAPD5, PDE4D, RASA1, RNF34, SCN3A, SP3
hsa-miR-19b-2*	8.65487785	DNAJA2, NME7, PCDH10, RHEBL1, SCN4B, ZDHHC7
hsa-miR-194*	9.33622365	ARHGAP21, CHD4, C11ORF9, DDEF1, DEPDC1B, HOOK3, HS3ST2, LPHN2, MGAT4A, MEIS2, MID1IP1, NUDC, PDHB, PHF1, RSBN1L, SNX1, YTHDF1
hsa-miR-141	0.15487933	ARPC5, CHD9, CXCL12, EPHA7, IPO5, KLF12, LMO3, MYH10, PRKACB, PLAG1, RANBP6, ZEB2, ZFR
hsa-miR-1233	0.15558856	DLGAP4, FMNL2, LRRC57, PDRG1, STIM1

by two or miRNAs in these target genes. When the target genes were analyzed, we noticed that parts of these genes play roles in the differentiation, proliferation, adhesion, apoptosis, and angiogenesis of tumor cells, indicating that these miRNAs are close related to tumor. For instance, KLF12 (target gene of hsa-miR-141) induces cell proliferation, angiogenesis and invasion in gastric cancer cell [24]. The depletion of PDRG1 (target gene of hsa-miR-1233) in colon cancer cells induces a decrease in cell proliferation [25]. RASA1 and PCDH10 (target genes of hsa-miR-223 and hsa-miR-19b-2* respectively) are also tumor suppressor genes [26, 27].

Although some of the miRNAs in gastric carcinoma, including hsa-miR-223 [15] and hsa-miR-141 [20], or in other types of carcinomas, have already been reported [28], some significantly altered miRNAs, such as hsa-miR-19b-2*, hsa-miR-194* and hsa-miR-1233, were newly discovered in gastric carcinoma in this study, which may serve as new biomarkers for gastric carcinoma diagnosis in the future. Besides, these abnormally expressed miRNAs might trigger or improve the tumorigenesis and progression of gastric carcinoma [12]. Further studies are still required to validate whether the microRNAs we selected can potentially function as either biomarkers or therapeutic targets and investigate their clinical significance and role in the development of gastric cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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hsa-miR-223:

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contribution	3' pairing contribution	local AU contribution	position contribution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	PCT
Position 439-446 of SP3 3' UTR hsa-miR-223	5' ...AUUUUUAUUAUGCAUAACUGACA... 3' ACCCCAUAAACUGU-UUGACUGU	8mer	-0.247	-0.029	-0.073	-0.003	-0.016	0.017	-0.35	97	4.124	0.47
Position 265-272 of FBXO8 3' UTR hsa-miR-223	5' ...UGUUAACGAAUGAUAAACUGACA... 3' ACCCCAUAAACUGUUUGACUGU	8mer	-0.247	0.003	-0.080	-0.047	-0.016	0.017	-0.37	97	1.742	0.47
Position 95-101 of SCN3A 3' UTR hsa-miR-223	5' ...AGGAGGUCCAUGCCAAACUGACU... 3' ACCCCAUAAACUGUUUGACUGU	7mer-m8	-0.120	0.003	-0.021	-0.048	-0.011	0.014	-0.18	79	1.936	0.48

hsa-miR-19b-2*

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contribution	3' pairing contribution	local AU contribution	position contribution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	PCT
Position 167-173 of NME7 3' UTR hsa-miR-19b	5' ...UGUUAGAACAUGGAUUUUGCACU... 3' AGUCAAAACGUACCU-AAACGUGU	7mer-m8	-0.120	-0.035	-0.056	-0.050	0.004	0.019	-0.24	88	1.655	0.50
Position 1207-1214 of SCN4B 3' UTR hsa-miR-19a	5' ...UUCUCCCAGAGCUGGUUUGCACA... 3' AGUCAAAACGUAUCUAAACGUGU	8mer	-0.247	0.003	0.119	0.191	0.008	0.025	> -0.03	1	1.669	0.70
Position 1207-1214 of SCN4B 3' UTR hsa-miR-19b	5' ...UUCUCCCAGAGCUGGUUUGCACA... 3' AGUCAAAACGUACCUAAACGUGU	8mer	-0.247	0.003	0.119	0.191	0.008	0.025	> -0.03	1	1.669	0.70
Position 141-148 of RHEBL1 3' UTR hsa-miR-19b	5' ...UGUGGCAUCCUCAUG--UUUGCACA... 3' AGUCAAAACGUACCUAAACGUGU	8mer	-0.247	-0.018	0.078	-0.079	0.008	0.025	-0.23	87	1.691	0.71
Position 141-148 of RHEBL1 3' UTR hsa-miR-19a	5' ...UGUGGCAUCCUCAUGUUUGCACA... 3' AGUCAAAACGUAUCUAAACGUGU	8mer	-0.247	-0.008	0.078	-0.079	0.008	0.025	-0.22	85	1.691	0.71

hsa-miR-194*

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contribution	3' pairing contribution	local AU contribution	position contribution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	PCT
Position 273-280 of CHD4 3' UTR hsa-miR-194	5' ...UCCCCACUGUAACGCCUGUUACA... 3' AGGUGUACCUCAACGACAAUGU	8mer	-0.247	0.003	0.094	-0.045	0.001	0.038	-0.16	81	1.583	0.49
Position 38-45 of ARHGAP21 3' UTR hsa-miR-194	5' ...CAAGUAAAAAAACUACUGUUACA... 3' AGGUGUACCUCAACGACAAUGU	8mer	-0.247	0.034	-0.027	-0.105	0.001	0.038	-0.31	97	1.065	0.21

hsa-miR-141

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contribution	3' pairing contribution	local AU contribution	position contribution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	PCT
Position 933-939 of ARPC5 3' UTR hsa-miR-200a	5' ...CCCCGUAAAUGUCUUCAGUGUUC... 3' UGUAGCAAUGGUCUGUCACAAU	7mer-m8	-0.120	0.003	-0.038	-0.006	0.015	0.020	-0.13	74	1.420	0.22
Position 933-939 of ARPC5 3' UTR hsa-miR-141	5' ...CCCCGUAAAUGUCUUCAGUGUUC... 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8	-0.120	0.012	-0.038	-0.006	0.015	0.020	-0.12	71	1.420	0.22
Position 1009-1015 of ARPC5 3' UTR hsa-miR-141	5' ...UUCUGUGUUUUAGCU--CAGUGUUU... 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8	-0.120	-0.025	-0.067	-0.016	0.015	0.020	-0.19	87	1.503	0.24
Position 1009-1015 of ARPC5 3' UTR hsa-miR-200a	5' ...UUCUGUGUUUUAGCU--CAGUGUUU... 3' UGUAGCAAUGGUCUGUCACAAU	7mer-m8	-0.120	-0.016	-0.067	-0.016	0.015	0.020	-0.18	86	1.503	0.24
Position 1617-1624 of CHD9 3' UTR hsa-miR-141	5' ...CCCUUAUUAGAGAGACAGUGUUA... 3' GGUAGAAAUGGUCU-GUCACAAU	8mer	-0.247	-0.018	-0.069	0.134	0.026	0.026	-0.15	79	1.095	0.26
Position 1617-1624 of CHD9 3' UTR hsa-miR-200a	5' ...CCCUUAUUAGAGAGACAGUGUUA... 3' UGUAGCAAUGGUCU-GUCACAAU	8mer	-0.247	-0.018	-0.069	0.134	0.026	0.026	-0.15	79	1.095	0.26

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 391-397 of LMO3 3' UTR hsa-miR-141	5' ...UGCAUUUAGUACAAUCAGUGUUU... 3' GGUAGAAAUGGUCU--GUCACAAU	7mer-m8	-0.120	-0.025	-0.112	-0.006	0.015	0.020	-0.23	92	2.073	0.50
Position 391-397 of LMO3 3' UTR hsa-miR-200a	5' ...UGCAUUUAGUACAAU-CAGUGUUU... 3' UGUAGCAAUGGUCUGUCACAAU	7mer-m8	-0.120	-0.016	-0.112	-0.006	0.015	0.020	-0.22	92	2.073	0.50
Position 608-614 of LMO3 3' UTR hsa-miR-200a	5' ...UGAGUUAGAGUCUAUCAGUGUUC... 3' UGUAGCAAUGGUCU---GUCACAAU	7mer-m8	-0.120	-0.016	-0.055	0.024	0.015	0.020	-0.13	76	1.459	0.23
Position 608-614 of LMO3 3' UTR hsa-miR-141	5' ...UGAGUUAGAGUCUAUCAGUGUUC... 3' GGUAGAAAUGGUCUGUCACAAU	7mer-m8	-0.120	-0.007	-0.055	0.024	0.015	0.020	-0.12	73	1.459	0.23

hsa-miR-1233

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 139-146 of DLGAP4 3' UTR hsa-miR-1233	5' ...AAAUUGACGCAUACAAGGGCUCA... 3' GACGCCCUCCUGUCCCGAGU	8mer	-0.247	0.013	-0.008	-0.079	0.000	-0.146	-0.47	99	2.540	N/A

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 111-118 of FMNL2 3' UTR hsa-miR-1233	5' ...ACAAAAAUAUUCUUAAGGGCUCA... 3' GACGCCCUCCUGUCCCGAGU	8mer	-0.247	0.045	-0.066	-0.086	0.000	-0.146	-0.50	99	1.386	N/A

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 1311-1317 of LRRC57 3' UTR hsa-miR-1233	5' ...GUGCCUUAUUGGUAGAGGGCUCU... 3' GACGCCCUCCUGUCCCGAGU	7mer-m8	-0.120	0.003	0.001	-0.027	-0.001	-0.085	-0.23	84	1.562	N/A

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 816-823 of PDRG1 3' UTR hsa-miR-1233	5' ...CUUAUGUGUUCAUUAAGGGCUCA... 3' GACGCCCUCCUGUCCCGAGU	8mer	-0.247	0.024	-0.093	-0.107	0.000	-0.146	-0.57	99	1.308	N/A

	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	site-type contri-bution	3' pairing contri-bution	local AU contri-bution	position contri-bution	TA contribution	SPS contribution	context+ score	context+ score percentile	conserved branch length	P_{CT}
Position 895-901 of STIM1 3' UTR hsa-miR-1233	5' ...CCACAGCCAUGAUACAGGGCUCU... 3' GACGCCCUCCUGUCCCGAGU	7mer-m8	-0.120	0.003	0.009	0.011	-0.001	-0.085	-0.18	71	1.508	N/A