Tumor-derived Exosome Promotes Metastasis via Altering its Phenotype and Inclusions

Running Title: Tumor-derived Exosomes are Evolutionary

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Supplementary Information

Experimental procedures

Cell culture

Human embryonic lung fibroblast (HELF) cells were cultured in DMEM (high glucose) medium. A549 and PC-9 cells were cultured in RPMI 1640 medium. All the medium were contain 10% exosome-depleted FBS and 1% penicillin-streptomycin. Cells lines were from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China).

Extraction of exosomes

Female BALB/C nude mice (four to six weeks old) were purchased from Jiesijie Animal center (Shanghai, China). All animal experiments were approved by our Animal Care and Use Committee to reduce the suffering and using of animals. No statistical method was used to pre-determine sample size. To establish an in situ lung cancer model, the mice were intrapulmonary implanted with A549 cells (1×10^6 cells/mice). The tumor-bearing mice were randomly divided into 2 groups (n=6). Group 1 was executed 14 days later after the injection, and group 2 was executed 35 days later after injection. The tumor tissues (500mg-1000mg/mouse) were carefully collected to avoid containing normal tissue, and tumor-derived exosomes were extracted similarly as described [1]. Briefly, tumor tissue was sliced into 1×1 mm pieces, and the pieces were placed into 10 ml 75 U/ml of collagenase type III. The tissue was then incubated in water bath at 37°C for 20 min. During the

incubation, the tube was shaken slowly. After that, the supernatant was collected and centrifuged at 200 g for 10 min, followed by 1,500 g for 10 min to remove debris. The collected medium was then ultracentrifuged at 100,000g for 2h at 4°C. The exosomes was washed with PBS, followed by another ultracentrifugation at for 2h at 4°C. Exosomes was collected for further characterization and experiments.

Characterization of exosomes

The size distribution of two exosomes samples were determined by nanoparticle tracking analysis (NTA; ZetaView, Germany). Samples were diluted in PBS to achieve a concentration between $1x10^8$-$2x10^9$/mL, and the results were measured with NTA. The surface appearance of two exosomes samples were measured by Multi Mode 8 Atomic Force Microscope (AFM; Bruker Co., Germany) and Transmission Electron Microscopy (TEM; JEM-1400plus, Japan). For AFM assay, 10 μL samples were added onto freshly cleaved mica and imaging was obtained by using AFM. To obtain the TEM images, 5-10 μL of E-exosome and A-exosome were dropped onto carbon-coated formvar Cu grids. Grids were washed with dd H2o and stained with uranyl acetate. The images were obtained with TEM. The expressions of the biomarkers (CD9 and CD63) were determined by flow cytometry and western blot.

Flow cytometry

E-exosome and A-exosome 30 μl ($1.4x10^7$ exosomes) were firstly incubated with 10 μL CD9 Flow Detection beads for 15 min at 25 °C, and then the mixture was diluted to 1 mL and

sharked for 30 min at 25 °C. After that, PBS (containing 100 mM glycine and 2% BSA) were used to stop the reaction. The mixture was centrifuged at 15,000 g for 5 min to remove the un-conjugated exosomes. After washing with PBS and blocking with 10% BSA, the result products were incubated with anti-CD9-FITC antibody (3 μl, Abcam) and anti-CD63-PE antibody (3 μl, Abcam) at 4°C for 1h, respectively. After the incubation, the products were centrifuged at 15,000 g for 5 min and washed with PBS. Background staining was performed by staining exosomes with isotype-matched control. Flow cytometric analysis was carried out on the BD FACSAriaIII (BD Biosciences), and the obtained data were analyzed with FlowJo software.

Western blot analysis

Western blot analysis was used to evaluate the expression of exosome surface markers CD9 and CD63, and performed as previously described protocols [2, 3]. Briefly, exosomes were lysed with RIPA lysis buffer (Thermo Fisher). The protein concentration was determined by using the BCA protein assay kit (Thermo Fisher). The equal qualities of protein were separated by SDS-PAGE (Bio-Rad), and transferred to PVDF membrane (Bio-Rad). The membranes were blocked in 5% skim milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 ℃. After washing, the membranes were incubated with the following secondary anti-bodies for 1h at room temperature. Chemiluminescent signals were generated by using a Super Signal West Pico Chemiluminescent Substrate kit (Pierce), and detected by using the ChemiDoc XPS system (Bio-Rad).

Small RNA High-throughput sequencing and data processing

Total RNA was extracted with TRIzol Reagent (Life Technologies) and quantified by using a Nanodrop and Qubit assay. Agilent 2100 Bioanalyzer was used to verify the integrity of RNA. Microarray experiments were performed at Beijing Weihui Biological Technology Co., Ltd. China. cDNA library was established with NEXTflex Small RNA-Seq Kit (Bioo Scientific). The raw sequencing reads were processed, followed by read extraction using high-throughput sequencing data analysis. The low quality reads (including the sequence of the unpredictable base sequence proportion over 10%, the sequence with 5' or 3' broken and poly A/T/G/C sequence) were abandoned for the accuracy of the results. The reads after screening are clean reads, and the final clean reads number and its proportion to total raw reads number were obtained. Demultiplexed RNA sequencing data were mapped against our curated human reference transcriptome to obtain miRNA raw read and frequency profiles. The mapped sRNA which can be matched to chromosomes in the genome were counted, and using Circos to map the distribution of reads on each chromosome. The mapped sRNAs were compared with the miRBase to obtain the sequence, secondary structure, length and frequency of matched miRNAs.

The ncRNA was also analysised by comparing the abundance of fragments of other RNA classes, such as tRNAs, snRNAs, snoRNAs, and rRNAs. The repeat classification was performed to avoid repeat reads. The sRNAs were compared to exons and introns of mRNA, and sRNA from mRNA degradation fragments was identified. New miRNA prediction and annotation were performed with miREvo [4] and miRDeep2 [5]. The iconic hairpin structure of the miRNA precursor was used to predict the novel miRNA in sRNA sequences.

Differential expression analysis of miRNA

Raw sequencing reads of predicted miRNAs were used for differential expression analysis. MicroRNA may undergo partial editing of bases, leading to changes in the sequence of seeds, resulting in altered target genes. By comparing the sRNA with the detected sequences of the known, new miRNA mature sequence and their precursors, the miRNA that may have been edited by the base mutation was founded. Statistics on the amount of expression of known and new miRNA in each sample were performed and normalized the expression quality with TPM. A miRNA expression difference analysis was conducted to detect miRNAs with significant differences in expression between E-exosome and A-exosome.

Prediction of miRNA targets

The target genes prediction for known miRNAs and novel miRNAs were conducted with miRanda software, and the relationship between miRNA and target genes was obtained.

Functional analysis of miRNA targets

Gene ontology (GO, http://www.geneontology.org/) term distribution and enrichment analysis were conducted, and enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified [6].

MiR-1260b inhibitor transfection

MiR-1260b inhibitor (Ambion) was transiently transfected into A549 cells with the siPORT

NeoFX transfection agent (Thermo Fisher) according to the manufacturer's instructions. Transfection of hsa-miR1260b inhibitor into A549 cells for 24 h, followed by incubation with A-exosome for 24 h. Then cell transwell and wound healing assays were performed.

Quantitative real-time PCR analysis

Total miRNA from exosomes were extracted using TRIzol Reagent (Life Technologies). These procedures were carried out as described previously [7, 8]. Quantitative real-time RT-PCR was performed in triplicate with an CFX96™ Real-Time PCR Detection System using TaqMan universal PCR master mix according to the manufacture's protocol (Bio-Rad). The TaqMan probes and primers were purchased from Thermo Fisher Scientific. RNU48 was used as endogenous control. The miR-1260b expression levels were determined using the 2-ΔΔCt method.

Effects of E-exosome and A-exosome on cancer metastasis

To determine the different effects of E-exosome and A-exosome on cancer metastasis, the expression of α-SMA on HELF cells after treated with E-exosome and A-exosome were measured by the immunofluorescence assay. Briefly, HELF cells were planted on confocal dishes and cultured at 37 °C for 24h. Then $1x10^9$ total E-exosome or A-exosome were added to the dishes and incubated at 37 °C for another 24h. The cells were washed with PBS (pH 7.4), and then treated with polyoxymethylene for 5 min. The cells were permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 5% BSA for 30 min. The cells were incubated with FITC-labeled anti-α-SMA (Abcam, ab8211; 1:100) at room

temperature for 90 min. After washing with PBS for 5 times, the cells were imaged with confocal fluorescence microscopy (Leica TCS SP8, Germany).

Transwell assay

We also determined the effects of exosomes in promoting cancer cell metastasis. A549 and PC-9 cells (1 x10^5 per well) were added into the upper chamber of transwell and medium contain 20% FBS were added into the lower chamber. After incubation with E-exosome and A-exosome (2 x10^8 per well) at 37 °C for 24 h, the migration cells were fixed with methanol and stained with crystal violet. Each sample was captured with 10 random visual fields with Carl Zeiss Primo Star microscopy (Carl Zeiss, Germany).

Wound healing assay

The migration ability of A549 cells was assessed in a classical wound healing assay. A549 cells were plated to six-well plates and treat with exosomes when they were attached. Then, monolayer cells were manually wounded by scraping the cells with a pipette tip and washed with PBS before the culture medium was replaced. After 24 h of incubation, progression of migration was observed and photographed by using an inverted microscope (Carl Zeiss, Germany).

Statistical analyses

Graphpad Prism version 5.01 was used for statistical analyses. Data are expressed as means ± standard deviations (SD). Statistical significance was determined using one-way

ANOVA or unpaired two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

References

1. Vella LJ, Scicluna BJ, Cheng L, Bawden EG, Masters CL, Ang CS, et al. A rigorous method to enrich for exosomes from brain tissue. Journal of extracellular vesicles. 2017; 6: 1348885.
2. Lu Y, Yu T, Liang H, Wang J, Xie J, Shao J, et al. Nitric oxide inhibits hetero-adhesion of cancer cells to endothelial cells: restraining circulating tumor cells from initiating metastatic cascade. Scientific reports. 2014; 4: 4344.
3. Lian S, Lu Y, Cheng Y, Yu T, Xie X, Liang H, et al. S-nitrosocaptopril interrupts adhesion of cancer cells to vascular endothelium by suppressing cell adhesion molecules via inhibition of the NF-small ka, CyrillicB and JAK/STAT signal pathways in endothelial cells. European journal of pharmacology. 2016; 791: 62-71.
4. Wen M, Shen Y, Shi S, Tang T. miREvo: an integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. BMC bioinformatics. 2012; 13: 140.
5. Friedlander MR, Mackowiak SD, Li N, Chen W, Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic acids research. 2012; 40: 37-52.
6. Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome biology. 2010; 11: R14.
7. Lu Y, Lian S, Ye Y, Yu T, Liang H, Cheng Y, et al. S-Nitrosocaptopril prevents cancer metastasis in vivo by creating the hostile bloodstream microenvironment against circulating tumor cells. Pharmacological research. 2019; 139: 535-49.
8. Hirata H, Hinoda Y, Shahryari V, Deng G, Tanaka Y, Tabatabai ZL, et al. Genistein downregulates onco-miR-1260b and upregulates sFRP1 and Smad4 via demethylation and histone modification in prostate cancer cells. British journal of cancer. 2014; 110: 1645-54.

Supplementary figures

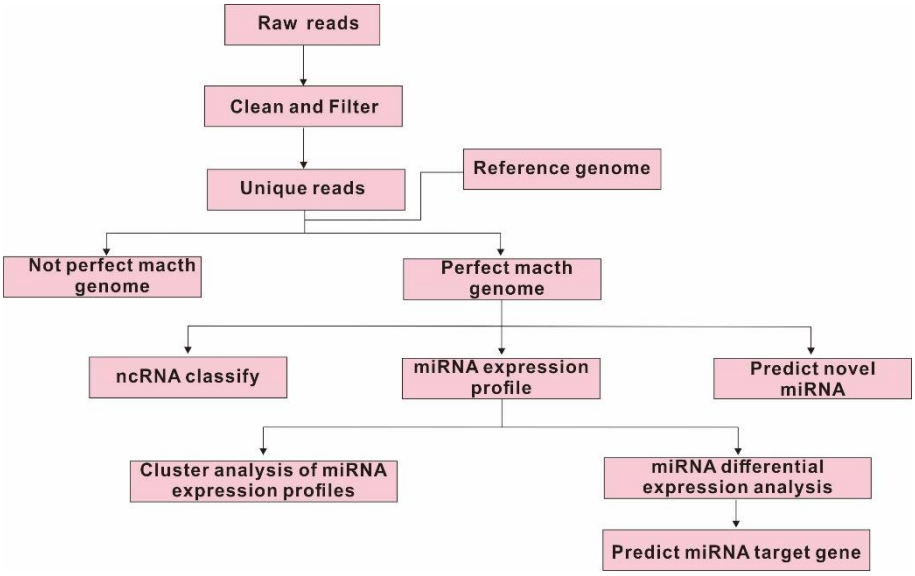

Figure S1. Schematic diagram of the miRNA analysis process.

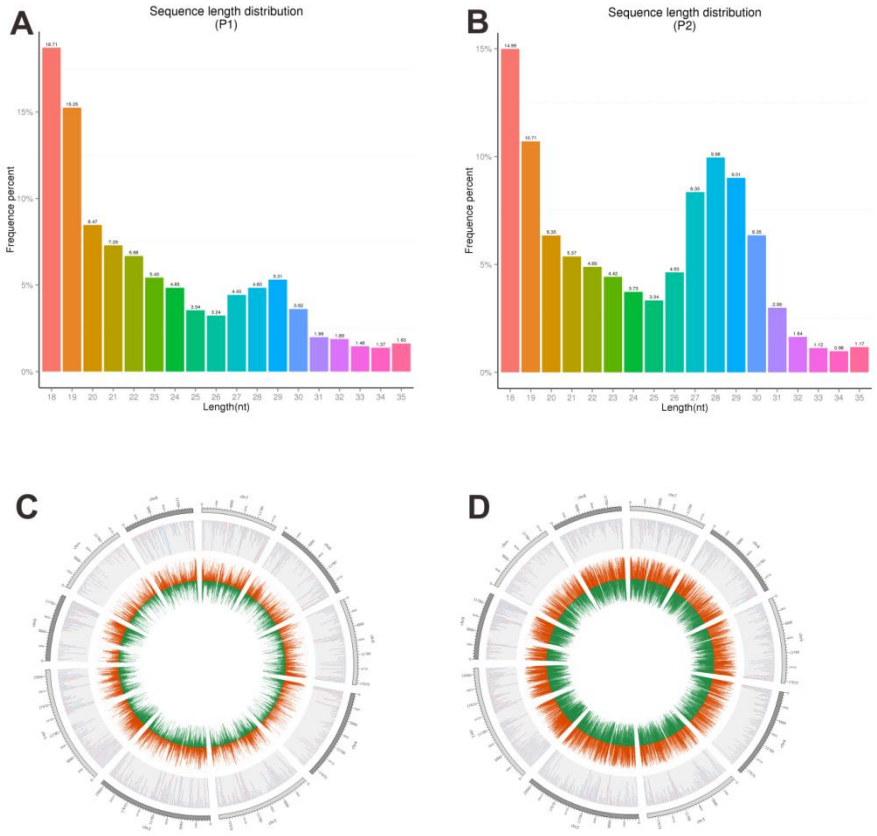

Figure S2. A-B,The sequence length distribution of sRNA in E-exosome(**A**) and A-exosome(**B**). **C-D,** Circos mapping show the distribution of reads on each chromosome. The outermost is the selection of displayed chromosomes. The grey background area in the

middle is the distributions of extracted 10000 reads, the red mapping to the positive chain, and the blue mapping to the negative chain. The innermost circles are all reads on the chromosome, orange is positively linked to coverage, and green is negative chain coverage distribution.

Supplementary tables

Table S1. List of RNA sequence filtering results

Sample	total reads	N%>10%	low quality	5 adapter contamine	3 adapter null or insert null	with ploy A/T/G/C	clean reads
E-exosome	22000000 (100.00%)	2696 (0.01%)	0(0.00%)	7075(0.03%)	1665472 (7.57%)	235782(1.07%)	20088975 (91.31%)
A-exosome	22000000 (100.00%)	2859 (0.01%)	0(0.00%)	6580(0.03%)	2089114 (9.50%)	251176(1.14%)	19650271 (89.32%)

Table S2. Comparison of sRNA with reference genome

Sample	Total sRNA	Mapped sRNA	+ Mapped sRNA	- Mapped sRNA
E-exosome	13029934(100.00%)	1873283(14.38%)	736317(5.65%)	1136966(8.73%)
A-exosome	10548350(100.00%)	1911922(18.13%)	784182(7.43%)	1127740(10.69%)

Table S3. Differential analysis results of miRNA expression

sRNA	E-exosome	A-exosome	log2.Fold_change.	p.value	Signature
novel_10	0	41403.33591	-16.337	0	TRUE
novel_113	0	17744.28682	-15.115	0	TRUE
novel_117	0	13012.477	-14.668	0	TRUE
novel_118	6699.30723	0	13.71	0	TRUE
novel_12	0	14195.42945	-14.793	0	TRUE
novel_125	64121.94063	42586.28836	0.59043	0	FALSE
novel_128	44024.01894	0	16.426	0	TRUE
novel_129	0	31939.71627	-15.963	0	TRUE
novel_134	3828.17556	0	12.902	0	TRUE
novel_137	1914.08778	0	11.902	0	TRUE
novel_139	8134.873066	0	13.99	0	TRUE
novel_140	0	33122.66873	-16.016	0	TRUE
novel_144	4306.697505	0	13.072	0	TRUE
novel_15	21054.96558	0	15.362	0	TRUE
novel_153	0	28390.85891	-15.793	0	TRUE
novel_163	11006.00474	0	14.426	0	TRUE
novel_168	0	4731.809818	-13.208	0	TRUE

novel_172	6699.30723	0	13.71	0	TRUE
novel_177	0	18927.23927	-15.208	0	TRUE
novel_181	0	22476.09664	-15.456	0	TRUE
novel_184	0	47318.09818	-16.53	0	TRUE
novel_190	10527.48279	0	14.362	0	TRUE
novel_192	18662.35586	0	15.188	0	TRUE
novel_194	0	28390.85891	-15.793	0	TRUE
novel_197	0	30756.76382	-15.909	0	TRUE
novel_199	0	7097.714727	-13.793	0	TRUE
novel_200	5742.26334	0	13.487	0	TRUE
novel_202	0	39037.431	-16.253	0	TRUE
novel_205	3349.653615	0	12.71	0	TRUE
novel_208	1914.08778	0	11.902	0	TRUE
novel_210	0	43769.24082	-16.418	0	TRUE
novel_222	0	20110.19173	-15.296	0	TRUE
novel_224	4785.21945	0	13.224	0	TRUE
novel_226	0	33122.66873	-16.016	0	TRUE
novel_227	0	30756.76382	-15.909	0	TRUE
novel_234	13877.13641	0	14.76	0	TRUE
novel_236	0	23659.04909	-15.53	0	TRUE
novel_245	26318.70698	0	15.684	0	TRUE
novel_247	0	36671.52609	-16.162	0	TRUE
novel_249	1914.08778	0	11.902	0	TRUE
novel_252	18183.83391	0	15.15	0	TRUE
novel_258	3349.653615	0	12.71	0	TRUE
novel_259	0	9463.619636	-14.208	0	TRUE
novel_261	3349.653615	0	12.71	0	TRUE
novel_262	0	7097.714727	-13.793	0	TRUE
novel_264	0	33122.66873	-16.016	0	TRUE
novel_267	6220.785285	0	13.603	0	TRUE
novel_269	0	16561.33436	-15.016	0	TRUE
novel_27	3828.17556	0	12.902	0	TRUE
novel_271	4785.21945	0	13.224	0	TRUE
novel_284	0	24842.00154	-15.6	0	TRUE
novel_308	0	20110.19173	-15.296	0	TRUE
novel_313	0	26024.954	-15.668	0	TRUE
novel_324	0	37854.47854	-16.208	0	TRUE
novel_329	20097.92169	0	15.295	0	TRUE
novel_33	0	30756.76382	-15.909	0	TRUE
novel_334	4785.21945	0	13.224	0	TRUE
novel_339	33975.0581	0	16.052	0	TRUE
novel_34	0	41403.33591	-16.337	0	TRUE
novel_343	0	18927.23927	-15.208	0	TRUE
novel_349	3349.653615	3548.857364	-0.083343	0	FALSE

novel_351	0	46135.14573	-16.494	0	TRUE
novel_357	0	3548.857364	-12.793	0	TRUE
novel_359	0	28390.85891	-15.793	0	TRUE
novel_365	6220.785285	0	13.603	0	TRUE
novel_373	2392.609725	0	12.224	0	TRUE
novel_378	2871.13167	0	12.487	0	TRUE
novel_383	19140.8778	0	15.224	0	TRUE
novel_394	0	67428.28991	-17.041	0	TRUE
novel_401	8134.873066	0	13.99	0	TRUE
novel_402	0	8280.667182	-14.016	0	TRUE
novel_408	0	17744.28682	-15.115	0	TRUE
novel_409	19619.39975	0	15.26	0	TRUE
novel_413	7177.829176	0	13.809	0	TRUE
novel_418	5742.26334	0	13.487	0	TRUE
novel_426	5742.26334	0	13.487	0	TRUE
novel_439	4306.697505	0	13.072	0	TRUE
novel_448	6220.785285	0	13.603	0	TRUE
novel_449	0	27207.90645	-15.732	0	TRUE
novel_457	0	20110.19173	-15.296	0	TRUE
novel_464	0	16561.33436	-15.016	0	TRUE
novel_467	0	17744.28682	-15.115	0	TRUE
novel_470	0	15378.38191	-14.909	0	TRUE
novel_474	0	18927.23927	-15.208	0	TRUE
novel_477	8613.395011	0	14.072	0	TRUE
novel_481	0	15378.38191	-14.909	0	TRUE
novel_487	0	14195.42945	-14.793	0	TRUE
novel_491	4785.21945	0	13.224	0	TRUE
novel_497	4785.21945	0	13.224	0	TRUE
novel_498	0	40220.38345	-16.296	0	TRUE
novel_502	7656.351121	0	13.902	0	TRUE
novel_512	0	57964.67027	-16.823	0	TRUE
novel_515	4785.21945	0	13.224	0	TRUE
novel_516	3349.653615	29573.81136	-3.1422	0	TRUE
novel_526	0	46135.14573	-16.494	0	TRUE
novel_53	3349.653615	0	12.71	0	TRUE
novel_538	0	14195.42945	-14.793	0	TRUE
novel_539	8613.395011	0	14.072	0	TRUE
novel_540	0	35488.57364	-16.115	0	TRUE
novel_544	0	18927.23927	-15.208	0	TRUE
novel_551	5263.741395	0	13.362	0	TRUE
novel_559	3828.17556	0	12.902	0	TRUE
novel_564	0	40220.38345	-16.296	0	TRUE
novel_57	0	17744.28682	-15.115	0	TRUE
novel_582	0	7097.714727	-13.793	0	TRUE

novel_585	0	8280.667182	-14.016	0	TRUE
novel_59	5263.741395	0	13.362	0	TRUE
novel_6	0	26024.954	-15.668	0	TRUE
novel_64	0	29573.81136	-15.852	0	TRUE
novel_67	21533.48753	0	15.394	0	TRUE
novel_7	1914.08778	0	11.902	0	TRUE
novel_73	2392.609725	0	12.224	0	TRUE
novel_78	11963.04863	0	14.546	0	TRUE
novel_82	0	35488.57364	-16.115	0	TRUE
novel_83	11963.04863	0	14.546	0	TRUE
novel_84	0	16561.33436	-15.016	0	TRUE
novel_87	5742.26334	0	13.487	0	TRUE
novel_88	18183.83391	0	15.15	0	TRUE
novel_89	6220.785285	0	13.603	0	TRUE
novel_157	1435.565835	0	11.487	########	TRUE
novel_253	1435.565835	0	11.487	########	TRUE
novel_434	1435.565835	0	11.487	########	TRUE
novel_86	1435.565835	0	11.487	########	TRUE
novel_119	0	2365.904909	-12.208	########	TRUE
novel_74	957.0438901	0	10.902	########	TRUE
hsa-miR-1260b	0	1182.952455	-11.208	########	TRUE
novel_100	0	1182.952455	-11.208	########	TRUE
novel_120	0	1182.952455	-11.208	########	TRUE
novel_196	0	1182.952455	-11.208	########	TRUE
novel_217	0	1182.952455	-11.208	########	TRUE
novel_341	0	1182.952455	-11.208	########	TRUE
novel_354	0	1182.952455	-11.208	########	TRUE
novel_360	0	1182.952455	-11.208	########	TRUE
novel_392	0	1182.952455	-11.208	########	TRUE
novel_395	0	1182.952455	-11.208	########	TRUE
novel_420	0	1182.952455	-11.208	########	TRUE
novel_431	0	1182.952455	-11.208	########	TRUE
novel_450	0	1182.952455	-11.208	########	TRUE
novel_455	0	1182.952455	-11.208	########	TRUE
novel_458	0	1182.952455	-11.208	########	TRUE
novel_488	0	1182.952455	-11.208	########	TRUE
novel_510	0	1182.952455	-11.208	########	TRUE
novel_558	0	1182.952455	-11.208	########	TRUE
novel_567	0	1182.952455	-11.208	########	TRUE
novel_92	0	1182.952455	-11.208	########	TRUE
hsa-miR-718	478.521945	0	9.9024	########	TRUE
novel_101	478.521945	0	9.9024	########	TRUE
novel_127	478.521945	0	9.9024	########	TRUE
novel_14	478.521945	0	9.9024	########	TRUE

novel_170	478.521945	0	9.9024	########	TRUE
novel_211	478.521945	0	9.9024	########	TRUE
novel_220	478.521945	0	9.9024	########	TRUE
novel_233	478.521945	0	9.9024	########	TRUE
novel_294	478.521945	0	9.9024	########	TRUE
novel_311	478.521945	0	9.9024	########	TRUE
novel_325	478.521945	0	9.9024	########	TRUE
novel_337	478.521945	0	9.9024	########	TRUE
novel_352	478.521945	0	9.9024	########	TRUE
novel_376	478.521945	0	9.9024	########	TRUE
novel_382	478.521945	0	9.9024	########	TRUE
novel_441	478.521945	0	9.9024	########	TRUE
novel_446	478.521945	0	9.9024	########	TRUE
novel_453	478.521945	0	9.9024	########	TRUE
novel_454	478.521945	0	9.9024	########	TRUE
novel_466	478.521945	0	9.9024	########	TRUE
novel_543	478.521945	0	9.9024	########	TRUE
novel_56	478.521945	0	9.9024	########	TRUE
novel_560	478.521945	0	9.9024	########	TRUE
novel_572	478.521945	0	9.9024	########	TRUE
novel_61	478.521945	0	9.9024	########	TRUE
novel_79	478.521945	0	9.9024	########	TRUE

Table S4. Novel miRNA sequence list

sRNA	**Sequence** (5’-3’)
>novel_10	ucgcgggcgcggggcaca
>novel_100	uccgcgggggcgggucccc
>novel_101	cccaggaggggcggcuc
>novel_113	ccccccuggggaaggcgg
>novel_117	cccgggccgggggcugcc
>novel_118	ccggcgggcgcggggcga
>novel_119	gccgggggcggcgggggac
>novel_12	gcccggcgggcggggcuc
>novel_120	cacaggguugguggaagg
>novel_125	gugcagcgcacgugcucc
>novel_127	cugcagcgcccggcgcaa
>novel_134	gcccggcuggggcggugg
>novel_137	cccaggcuggggcggggc
>novel_139	cuccaggggaaggggcccc
>novel_14	ccguaccccggggccgcuc
>novel_140	caggccggcggggacaac
>novel_144	cccgccgcgggggugggg

>novel_15 acccugggucggggcagc
>novel_153 cgccgggcggcggggcug
>novel_157 cagcgagggcugggcugac
>novel_163 cacagaggggugggugggc
>novel_168 ccagggcggcggggcgcg
>novel_170 cucagagagaggggcaagu
>novel_172 ccggggcccggguugggg
>novel_177 gccaggcccuggcggggauu
>novel_181 uuccagggcugggucucug
>novel_184 cuucaccgccugggcuuc
>novel_190 cccugaagaggggguagg
>novel_192 caggagggagggaacugac
>novel_194 ccgcagcgggcgggggca
>novel_196 cgccgccggcggggccac
>novel_197 cuucagagaggggcccuucc
>novel_199 ccccagggccccgcaccc
>novel_200 cccagaagaggggccuc
>novel_202 acucgagccgggcgcggu
>novel_205 cccgcgggugggguggguu
>novel_208 cccagggugggggcucu
>novel_210 guguagggcugggugcgg
>novel_211 cacggggaggcccgggaac
>novel_217 aagcgcagggcccggagc
>novel_220 aacaagcacccggccuccc
>novel_222 ugccaagggcagggcagg
>novel_224 ccuaggguggggguggg
>novel_226 ccgccgcggcgggggug
>novel_227 ccucaggaaggggauucc
>novel_233 ccagagggcugcgucucac
>novel_234 uagcagggcugggugccc
>novel_236 gguuucccggcggcguccc
>novel_245 ccccagagaggggcccgucuc
>novel_247 cccugggucggggcuguc
>novel_249 gcccgggcuggagaggugg
>novel_252 cuccaccccgcggacguc
>novel_253 agacaggggcccgggacac
>novel_258 cuccaggcaggaggaugu
>novel_259 cgcagggagcgggggugc
>novel_261 aaccagggcuggguguga
>novel_262 cgccaagggcaggggagc
>novel_264 gacaaagggcagggacuc
>novel_267 agcuggaggcugaggguag
>novel_269 gcguagggcuggguaggg
>novel_27 cagagcgaggggguccg

>novel_271 ccuuggggaaggggguca
>novel_284 cagcaggggcuggggcua
>novel_294 caaggagagaggggguc
>novel_308 acagaagggcuggggagg
>novel_311 cccggagaggggccuugg
>novel_313 gcccagggcuggguagaggc
>novel_324 ccgagggcgcggggaugc
>novel_325 aaagagggcuggguccugc
>novel_329 ccuagagagaggggagac
>novel_33 ccggggcuggggcggucc
>novel_334 cacucccggugagcucuggg
>novel_337 ucaagagagaggggcuug
>novel_339 ggccgggcgugguggagac
>novel_34 ggccggccgccggacccc
>novel_341 agccaagggacggggacc
>novel_343 cagccuggcuggagcuug
>novel_349 gcgccgggcgcggggagac
>novel_351 cccgccgcgggggcucuc
>novel_352 ccggcggcccggacccuc
>novel_354 cugcagggcuggguugca
>novel_357 ccaguuucuggcaccugac
>novel_359 ccagagggcugggucaug
>novel_360 acaagagggagggguccc
>novel_365 ccagaagggcaggggagu
>novel_373 cucagagagaggggucag
>novel_376 ccuugaggaggggggaggag
>novel_378 ccucaagaggggagaua
>novel_382 ccagccccggccggucca
>novel_383 cccugggugggggcucc
>novel_392 ucccuuugggaggcaggg
>novel_394 ccagagggcugggugagg
>novel_395 acaaagggcuggguacaa
>novel_401 cccagggugggggaugg
>novel_402 ugccaagggcagggaugc
>novel_408 gagcaggggacggggac
>novel_409 caucgggcuggagaggguc
>novel_413 cccagccuggggaggccc
>novel_418 ccuggagagaggggcucug
>novel_420 ccaggggcuggguuugg
>novel_426 cccuggggagggggaugu
>novel_431 aaagagagggaaggcaca
>novel_434 caggggucccggguucugg
>novel_439 cccugaagaggggauuuuc
>novel_441 ggcccugcccucugggcac

>novel_446 augcagggcugggucugc
>novel_448 ccaggaccgccuggggggua
>novel_449 cccugaagaggggggaugu
>novel_450 cacaaagggaugggggaug
>novel_453 ggccauguagaggcaggg
>novel_454 gcagaggcugggcagugcc
>novel_455 gcccaagggacgggaggg
>novel_457 aaagaggcgggagauuca
>novel_458 cacggucacaguggcuccc
>novel_464 gaguggaggggggaaagag
>novel_466 cauacggggcugggccuc
>novel_467 cuaugggucgggcgcggug
>novel_470 cuccaggcugaggccuca
>novel_474 aaaggaggcugaggcuua
>novel_477 ccacaagggcagggcuca
>novel_481 gugggggcuggagagaggaa
>novel_487 caguagggcugggugcag
>novel_488 ucuggggucuguagcaaac
>novel_491 cccagggcuggagagggag
>novel_497 cccggggugggggugcg
>novel_498 uuccaagggcagggcacc
>novel_502 cccuggggugggggcagcc
>novel_510 gacucgggucgggguccc
>novel_512 cccaaggccuacccacac
>novel_515 cucaaagaggggagggg
>novel_516 ccacgaagaggggagug
>novel_526 gccagcguuggugggуga
>novel_53 gccugggagggcgcgggu
>novel_538 cagcgcguuuguggguag
>novel_539 cccaagagaggggaggaa
>novel_540 aacuaagggcaggggaug
>novel_543 guuugggcuagagaucug
>novel_544 aauugucuggagaagggca
>novel_551 ccucagagaggggcugcc
>novel_558 cugcagguuggggauugc
>novel_559 cagaaagggcaggggcag
>novel_56 cccgaggcgggggaaccg
>novel_560 accaggagauggggugaggg
>novel_564 cagggaccagccuggagac
>novel_567 aacagcgugggugggaua
>novel_57 ccgcgccggcgggaggagg
>novel_572 gcuuagggcugggugccg
>novel_582 ccgcggagcgggagcgcg
>novel_585 gcagggcuggggcggagg

>novel_59	cgcgggcggggcgggugg
>novel_6	cgccggcggcggggcagc
>novel_61	cccgggcggcggcgacac
>novel_64	agcgcccgcgggggucgu
>novel_67	ugccgggugggcggcccc
>novel_7	caccagcgcccggcgcgu
>novel_73	cgccgaggggggcgggggga
>novel_74	cgcaccccagccucccuc
>novel_78	cgaggaagcccggcugcc
>novel_79	uaagagcgcccgggcuucc
>novel_82	ggagaaggggaggggggcc
>novel_83	ccgugggggugggggagc
>novel_84	gcccggacgggcgaguca
>novel_86	cgcgcccggcggcggcug
>novel_87	ccggacccggcggcgaggg
>novel_88	ccccagcgggcgggcggcagc
>novel_89	cuccgcggcgggggcgcc
>novel_92	cccgggaggggcgcgcc

Table S5. Target gene prediction results

miRNA	target_mRNA	target_gene
hsa-miR-1260b	ENST00000000412	ENSG00000003056
hsa-miR-1260b	ENST00000000442	ENSG00000173153
hsa-miR-1260b	ENST00000002829	ENSG00000001617
hsa-miR-1260b	ENST00000003583	ENSG00000001460
hsa-miR-1260b	ENST00000003912	ENSG00000001461
hsa-miR-1260b	ENST00000004531	ENSG00000003989
hsa-miR-1260b	ENST00000005178	ENSG00000004799
hsa-miR-1260b	ENST00000006015	ENSG00000005073
hsa-miR-1260b	ENST00000006053	ENSG00000006210
hsa-miR-1260b	ENST00000006275	ENSG00000007255
hsa-miR-1260b	ENST00000006777	ENSG00000005486
hsa-miR-1260b	ENST00000007510	ENSG00000004777
hsa-miR-1260b	ENST00000007633	ENSG00000006534
hsa-miR-1260b	ENST00000009180	ENSG00000010278