Genetic Polymorphisms in *miR-604*A>G, *miR-938*G>A, *miR-1302-3*C>T and the Risk of Idiopathic Recurrent Pregnancy Loss

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Supplementary Table S1

FSH, LH and E2 levels of miRNA polymorphic genotypes in Control and RPL patients

Genotypes	FSH(mIU/mL)		LH(mIU/mL)		E2(pg/mL)	
	Control(113)	Case (388)	Control(113)	Case (388)	Control(113)	Case (388)
miR-604A>G						
AA	8.18 ± 10.48	7.17 ± 9.27	3.05 ± 4.08	35.10 ± 32.00	31.11 ± 283.40	5.29 ± 5.370
AG	8.26 ± 7.95	7.95 ± 12.29	3.36 ± 2.24	36.67 ± 28.49	22.88 ± 173.07	7.16 ± 16.61
GG	7.55 ± 4.53	6.91 ± 5.67	3.68 ± 3.36	33.34 ± 22.45	25.45 ± 166.34	7.95 ± 6.46
***P*[a]**	0.643	0.854	0.436	0.899	0.031	0.592
miR-608C>G						
CC	7.704 ± 4.10	5.75 ± 2.68	3.43 ± 1.96	4.33 ± 2.19	27.11 ± 13.92	40.07 ± 39.66
CG	7.93 ± 10.41	7.70 ± 11.59	3.17 ± 3.22	5.71 ± 7.11	28.04 ± 267.95	34.16 ± 26.92
GG	8.61 ± 7.50	8.80 ± 12.64	8.96 ± 9.93	9.26 ± 21.39	22.62 ± 174.45	34.67 ± 22.80
***P*[a]**	0.4	0.351	0.731	0.103	0.215	0.561
miR-631I/D						
II	8.15 ± 7.43	7.68 ± 10.95	3.30 ± 3.12	6.53 ± 12.61	27.27 ± 215.84	35.84 ± 30.25
ID	7.9 ± 12.93	5.80 ± 3.07	3.42 ± 2.70	3.79 ± 1.63	18.38 ± 169.96	34.36 ± 21.13
DD	NA	NA	NA	NA	NA	NA
p	0.743	0.351	0.796	0.373	0.025	0.849
miR-938G>A						
GG	8.16 ± 8.49	7.11 ± 8.97	3.36 ± 3.28	6.11 ± 11.84	25.43 ± 205.77	35.73 ± 29.54
GA	7.36 ± 1.36	27.03 ± 39.53	2.44 ± 0.75	15.16 ± 21.68	35.83 ± 381.45	34.2 ± 30.07
AA	NA	NA	NA	NA	NA	NA
***P*[a]**	0.743	< 0.001	0.25	0.139	0.093	0.929
miR-1302-3C>T						
CC	7.86 ± 7.16	7.77 ± 11.11	3.31 ± 3.28	6.52 ± 12.78	26.00 ± 226.10	35.30 ± 30.13
CT	10.05 ± 11.91	5.59 ± 3.26	3.33 ± 1.37	4.34 ± 3.45	25.93 ± 165.76	39.78 ± 21.70
TT	NA	NA	NA	NA	NA	NA
***P*[a]**	0.009	0.643	0.244	0.702	0.988	0.576

Abbreviations: RPL, recurrent pregnancy loss; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; E2, Estradiol; PLT, platelet count; SD, standard deviation.

[a]One-way analysis of variance test. [b]Mean ± SD.

Supplementary Table S2
Clinical variables in RPL patients, stratified by microRNA polymorphism status

Genotypes	CD56+ NK Cells (%)	T. chol (mg/dl)	PT (sec)	PAI-1 (ng/ml)	Uric acid (mg/dl)	PLT (10^3/ul)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
miR-604A>G						
AA	17.608 ± 7.501	184.914 ± 49.466	11.643 ± 0.868	11.263 ± 5.961	3.728 ± 0.767	256.337 ± 66.487
AG	18.678 ± 8.503	189.543 ± 51.960	11.561 ± 0.865	10.172 ± 5.677	3.851 ± 0.917	253.168 ± 54.183
GG	18.467 ± 7.453	192.278 ± 37.644	11.400 ± 0.786	9.158 ± 4.889	3.894 ± 0.756	261.857 ± 49.529
***P*[a]**	0.769	0.771	0.495	0.383	0.578	0.754
miR-608C>G						
CC	19.159 ± 9.643	185.627 ± 45.265	11.563 ± 1.012	10.091 ± 5.322	3.629 ± 0.701	260.520 ± 58.040
CG	17.780 ± 7.322	189.772 ± 54.521	11.595 ± 0.836	10.431 ± 5.766	3.921 ± 0.917	261.321 ± 62.694
GG	18.372 ± 7.864	185.568 ± 41.961	11.569 ± 0.759	11.353 ± 6.254	3.724 ± 0.755	237.510 ± 49.367
***P*[a]**	0.740	0.853	0.970	0.677	0.121	0.051
miR-631I/D						
II	18.066 ± 7.996	187.707 ± 49.780	11.576 ± 0.866	10.598 ± 5.648	3.796 ± 0.830	255.700 ± 59.949
ID	20.600 ± 7.905	188.077 ± 46.320	11.633 ± 0.796	9.976 ± 6.517	3.854 ± 0.935	252.067 ± 50.730
P[a]	0.336	0.979	0.788	0.703	0.81	0.820
miR-938G>A						
GG	18.345 ± 7.970	186.909 ± 49.650	11.586 ± 0.861	10.456 ± 5.685	3.801 ± 0.844	255.81 ± 59.788
GA	14.333 ± 9.504	216.600 ± 31.214	11.350 ± 0.751	20.000	3.750 ± 0.311	240.400 ± 27.199
***P*[a]**	0.392	0.186	0.588	0.097	0.904	0.567
miR-1302-3C>T						
CC	18.000 ± 7.847	189.463 ± 51.042	11.583 ± 0.882	10.427 ± 5.618	3.814 ± 0.851	254.239 ± 59.177
CT	21.800 ± 9.545	174.579 ± 31.964	11.569 ± 0.620	12.354 ± 6.895	3.678 ± 0.721	263.400 ± 60.440
TT	NA	161.000	NA	4.370	3.800	316.000
***P*[a]**	0.169	0.402	0.945	0.334	0.809	0.479

Abbreviations: RPL, recurrent pregnancy loss; T. chol, Total cholesterol; PAI-1, plasminogen activator inhibitor-1; PT, prothrombin time; PLT, platelet count; SD, standard deviation.

[a]One-way analysis of variance test.

Supplementary Table S3

Clinical variables in RPL patients with pregnancy loss ≥3 stratified by microRNA polymorphism status

Genotypes	CD56+ NK Cells (%) Mean ± SD	T. chol (mg/dl) Mean ± SD	PT (sec) Mean ± SD	PAI-1 (ng/ml) Mean ± SD	Uric acid (mg/dl) Mean ± SD	PLT (10^3/ul) Mean ± SD
miR-604A>G						
AA	17.425 ± 7.928	189.757 ± 54.029	11.657 ± 0.816	10.795 ± 5.726	3.844 ± 0.855	244.825 ± 57.931
AG	18.203 ± 9.308	191.179 ± 61.182	11.672 ± 0.944	9.958 ± 4.161	3.759 ± 0.818	256.830 ± 51.186
GG	17.625 ± 9.211	181.200 ± 31.247	11.440 ± 0.690	9.617 ± 6.041	4.010 ± 0.886	258.250 ± 38.805
P[a]	0.927	0.879	0.738	0.810	0.691	0.525
miR-608C>G						
CC	18.695 ± 10.468	178.655 ± 51.508	11.602 ± 0.959	9.397 ± 4.860	3.733 ± 0.568	243.571 ± 49.083
CG	17.418 ± 7.719	199.268 ± 60.245	11.653 ± 0.827	10.826 ± 5.018	4.079 ± 0.976	257.840 ± 58.988
GG	17.677 ± 8.617	183.625 ± 44.412	11.673 ± 0.850	11.593 ± 6.817	3.313 ± 0.515	250.048 ± 40.169
P[a]	0.860	0.275	0.952	0.524	0.012	0.511
miR-631I/D						
II	17.678 ± 8.707	189.654 ± 56.148	11.668 ± 0.854	10.409 ± 5.154	3.829 ± 0.828	252.739 ± 53.254
ID	19.500 ± 7.969	185.400 ± 34.004	11.338 ± 0.968	9.985 ± 5.800	3.760 ± 1.046	244.429 ± 46.669
P[a]	0.621	0.868	0.300	0.852	0.860	0.689
miR-938G>A						
GG	17.891 ± 8.584	189.407 ± 54.978	11.643 ± 0.863	10.361 ± 5.172	3.825 ± 0.835	252.152 ± 52.643
GA	14.500 ± 13.435	NA	NA	NA	NA	NA
P[a]	0.586	NA	NA	NA	NA	NA
miR-1302-3C>T						
CC	17.692 ± 8.453	191.922 ± 57.489	11.670 ± 0.887	9.794 ± 4.830	3.845 ± 0.863	251.261 ± 51.729
CT	19.640 ± 12.015	167.889 ± 11.677	11.438 ± 0.636	17.307 ± 4.572	3.656 ± 0.559	259.273 ± 61.772
TT	NA	NA	NA	NA	NA	NA
P[a]	0.627	0.217	0.383	0.004	0.524	0.637

Abbreviations: RPL, recurrent pregnancy loss; T. chol, total cholesterol; PAI-1, plasminogen activator inhibitor-1; PT, prothrombin time; PLT, platelet count; SD, standard deviation. Statistically significant values are indicated in red.

[a] One-way analysis of variance test.

Supplementary Table S4

Plasma folate and homocysteine levels according to miRNA polymorphic genotype in RPL patients with pregnancy loss ≥2 and ≥3

	PL≥2	PL≥3	PL≥2	PL≥3
Genotypes	**Folate (mg/ml)**		**Hcy (mmol/L)**	
miR-604A>G				
AA	14.604 ± 15.832	13.973 ± 16.770	7.136 ± 2.414	7.198 ± 2.152
AG	13.579 ± 8.221	14.487 ± 9.712	6.881 ± 1.710	6.981 ± 1.802
GG	15.709 ± 9.085	14.948 ± 9.217	6.744 ± 2.367	6.370 ± 1.693
P[a]	0.690	0.964	0.523	0.323
miR-608C>G				
CC	12.771 ± 8.192	12.868 ± 8.964	7.375 ± 2.651	7.241 ± 2.216
CG	14.142 ± 12.870	15.025 ± 15.841	6.855 ± 1.847	7.045 ± 1.932
GG	15.593 ± 12.845	14.568 ± 10.727	6.843 ± 1.953	6.720 ± 1.683
P[a]	0.469	0.746	0.202	0.472
miR-631I/D				
II	14.551 ± 12.274	14.817 ± 13.326	6.945 ± 2.133	6.971 ± 1.948
ID	10.092 ± 5.317	8.106 ± 1.857	7.330 ± 1.578	7.741 ± 1.975
P[a]	0.139	0.135	0.409	0.228
miR-938G>A				
GG	14.291 ± 12.021	14.450 ± 13.019	6.961 ± 2.091	6.981 ± 1.935
GA	9.625 ± 3.686	6.610 ± 0.693	7.947 ± 2.506	10.000 ± 0.566
P[a]	0.440	0.398	0.351	**0.029**
miR-1302-3C>T				
CC	14.110 ± 11.892	14.651 ± 12.848	6.989 ± 2.155	7.004 ± 1.986
CT	14.846 ± 12.808	11.563 ± 14.006	6.812 ± 1.426	7.160 ± 1.595
TT	19.120	NA	7.490	NA
P[a]	0.885	**0.006**	0.895	0.776

Abbreviations: RPL, recurrent pregnancy loss; Hcy, homocysteine; SD, standard deviation. Statistically significant values are indicated in red.

[a] One-way analysis of variance test.

Supplementary Table S5
Prediction of microRNA target genes

microRNA	Target gene	Protein product
miR-604	MTHFR	Methylenetetrahydrofolate reductase
miR-608	IGFBP5	insulin-like growth factor binding protein 5
	EIF5A	eukaryotic translation initiation factor 5A
	TNFAIP1	tumor necrosis factor, alpha-induced protein 1
miR-631	TGFB3	transforming growth factor, beta 3
	TIMP2	TIMP metallopeptidase inhibitor 2
	IGF1R	insulin-like growth factor 1 receptor
miR-938	GNRHR	gonadotropin-releasing hormone receptor
	TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)
miR-1302-3	TRAF4	TNF receptor-associated factor 4

Supplementary Table S6
Location & host gene name of miRNA

miRNA name	Location(chromosome)	host gene
miR-604	10p11.23	SVIL-203(intron 9), Supervillin
miR-608	10q24.31	SEMA4G(intron 3), Semaphorin-4G
miR-631	15q24.2	NEIL1(intron 5), Endonuclease VIII-like 1
miR-938	10p11.23	SVIL-201(intron 3), Supervillin
miR-1302-3	2q13	intergenic

Supplementary Table S7
Primer, PCR condition, enzyme information for PCR-RFLP

miRNA	primers
miR-604	F:CTT GGC TCA GTG GTC TGT TT
	R:GTA CAG GGA CTG AAA GGT GAA G
miR-608	F:GTG GGT CAC ACT TGT AAT CT
	R:AAT TCT GAG GGT GTT CAC TG
miR-631	AAT CCC ACT CCA GGA TGG GAA A
	TGA CAG AGG AAC AGG CAG AGA T
miR-938	T GGT GCA CTG GGT TCA CCT TTA AGC G
	GTA ATA CCT CTG AGC CTT TGG GGC C
miR-1302-3	AAC TAA GCT TGG GAA ATA TTT ATG CCA
	GAG CAT CAT CAG TCC AAA GTC C

PCR condition	
initial denaturation	95°C for 5 min
denaturation	95°C for 30 sec
annealing	59°C for 30 sec
extension	72°C for 30 sec
final extension	72°C for 5 min
total cycles	35 cycles

restriction enzymes

miR-604	*Bss*SI
miR-608	*Pvu*II
miR-631	*Nla*IV
miR-938	*Hha*I
miR-1302-3	*Nla*III

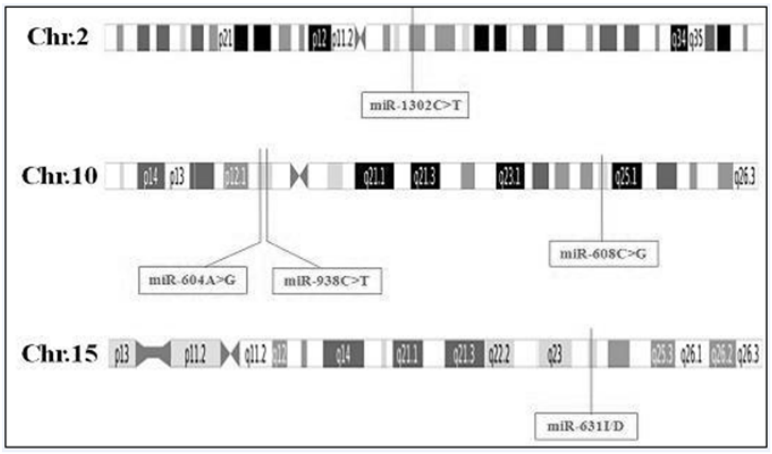


Figure S1: The respective loci of the five miRNAs analyzed in this study (miR-604/miR-608/miR-631/miR-938/miR-1302-3), present on chromosomes 2, 10, and 15.

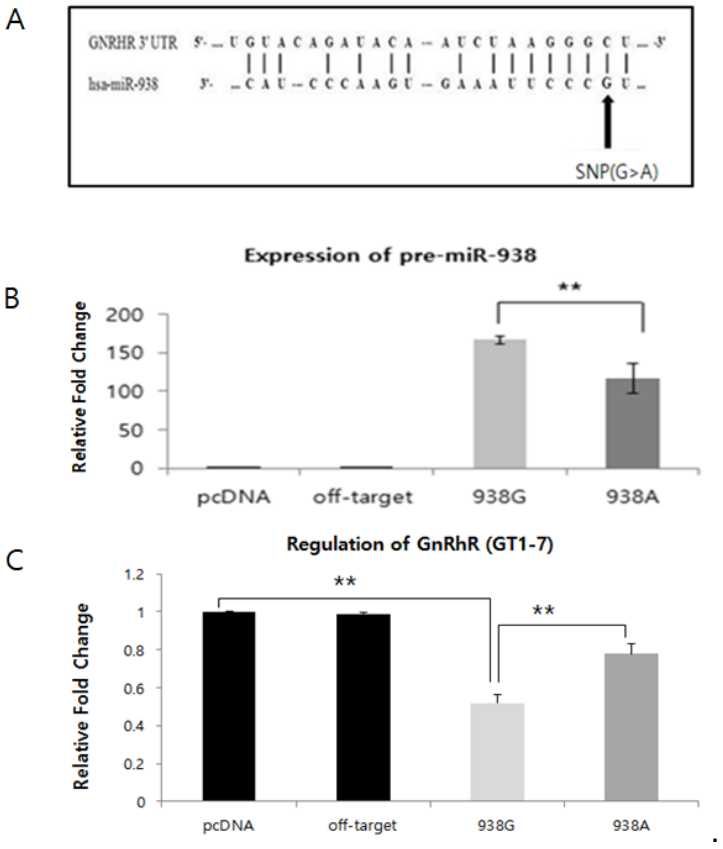


Figure S2. The miRNA, miR-938, regulates GnRHR mRNA via a targeting sequence located in the 3′ UTR of this gene. A. A schematic representation of putative target genes with 3′-UTRs that contain possible miR-938-G and miR-938-A binding sites in conserved regions. B. Expression of miR-938G>A. Levels of miR-938 were detected by miRNA reverse transcriptase quantitative PCR (qRT-PCR) in cells transfected with the empty pCR3.1 vector, pCR3.1-miR-offtarget, pCR3.1-miR-938-GG, or pCR3.1-miR-938-AA. U6 snRNA was used as an internal control, with the relative level of miR-938 normalized to U6. **P<0.05. C. Dual-luciferase reporter assays to measure the strength of interaction between miR-938 and its target sequence in the GnRHR 3′UTR. Reporter constructs contain the predicted targeting sequence for the GnRHR gene (pGL4.13-GnRHR 3′UTR) cloned into the 3′UTR of

the reporter gene. Data represent three independent experiments with triplicate measurements. **P <0.05.