Association between microRNA machinery gene polymorphisms and recurrent implantation failure

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Abstract. The present study aimed to investigate the potential association of five miRNA machinery gene polymorphisms (*DICER1* rs3742330A>G, *DROSHA* rs10719T>C, *RAN* rs14035C>T, *XPO5* rs11077A>C and *DGCR8* rs417309G>A) with recurrent implantation failure (RIF), a clinical condition in which good-quality embryos repeatedly fail to implant following two or more *in vitro* fertilization cycles, and its associated risk factors in Korean women. Therefore, the present study performed genotype analysis and assessed the frequency of these miRNA gene polymorphisms in patients diagnosed with RIF (n=119) and randomly selected controls (n=210) with at least one live birth and no history of pregnancy loss. The *DROSHA* rs10719T>C and *RAN* rs14035C>T polymorphisms were identified to be significantly associated with decreased prevalence of RIF. Additionally, the *DROSHA* rs10719 TC and the *RAN* rs14035 CT genotypes were present at significantly lower frequencies in the RIF group than in the control group (adjusted odds ratio=0.550; 95% CI, 0.339-0.893; P=0.016; and adjusted odds ratio=0.590; 95% CI, 0.363-0.958; P=0.033, respectively). Furthermore, the combined *RAN* rs14035 CT+TT genotype was observed to be associated with decreased RIF prevalence (adjusted odds ratio=0.616; 95% CI, 0.386-0.982; P=0.042). Genotype combination analysis for the various miRNA polymorphisms revealed that the *DROSHA* TC genotype exhibited a highly significant negative association with RIF prevalence when combined with the *RAN* CT genotype (adjusted odds ratio=0.314; 95% CI, 0.147-0.673; P=0.003; false discovery rate-adjusted P=0.023). The present study revealed an association between the *DROSHA* rs10719 and *RAN* rs14035 3'UTR polymorphisms and decreased risk of RIF in Korean women, which suggests that these gene polymorphisms could represent potential markers for predicting RIF risk.

Introduction

Implantation failure is the most frequent cause of pregnancy loss after *in vitro* fertilization (IVF) and embryo transfer (ET), and a substantial number of patients fail to achieve successful pregnancy, even after repeated IVF attempts (1). Recurrent implantation failure (RIF) refers to a clinical condition in which good-quality embryos repeatedly fail to implant following two or more IVF cycles (1). The precise definition, however, remains controversial, and different fertility centers practicing IVF may use different definitions for this condition (2,3). Despite various known causes of RIF, including embryonic or uterine factors, cytogenetic abnormalities, genetics, and immunologic factors, the etiology of RIF remains undetermined in most cases, and this presents a major challenge for both clinicians and researchers (2).

One potential molecular mechanism that may influence embryo implantation is regulation of microRNAs (miRNAs) (4). These are an abundant set of evolutionarily conserved small RNAs that control posttranscriptional gene expression by targeting messenger RNAs (mRNAs) for degradation, translational repression, or both (5,6). Functionally, miRNAs exert their activity by binding to the 3'-untranslated region (UTR) of their target mRNA, thereby repressing gene expression. A single miRNA can regulate multiple target mRNAs simultaneously, thereby acting as a master regulator of gene expression and cellular function. Therefore, miRNAs play pivotal roles in both physiological and pathological processes (5).

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In a cell, miRNA biosynthesis involves the activity of several miRNA machinery genes in multiple steps. RNA polymerase II mediates transcription of primary miRNA transcripts (pri-miRNAs) in the nucleus. The endonuclease Drosha ribonuclease III (DROSHA) and its cofactor, DiGeorge syndrome critical region gene 8 (DGCR8), then cleave this long pri-miRNAs to form precursor-miRNAs (pre-miRNA). This pre-miRNA is exported from the nucleus to the cytoplasm by the combined activity of Ras-related nuclear protein (RAN) GTPase and exportin 5 (XPO5), and is then converted into mature miRNA by Dicer 1, ribonuclease III (DICER1) (7). These miRNA machinery genes are located in chromosome 5 (*DROSHA*), chromosome 14 (*DICER1*), chromosome 12 (*RAN*), chromosome 6 (*XPO5*), and chromosome 22 (*DGCR8*) (8-12).

The miRNA machinery proteins must function precisely to generate correct miRNAs, in order for these species to perform their numerous essential roles accurately. Abnormal expression of even a single miRNA can have a detrimental impact on the outcome of diverse cellular functions via modulation of target gene activity, and there is increasing evidence for deregulated miRNA expression in a variety of reproductive disorders, including endometriosis, leiomyoma, and implantation failure (4). In particular, Revel *et al* (13) reported altered miRNA expression in the secretory endometrium of RIF patients, as compared to fertile women, indicating that aberrantly expressed miRNAs may contribute to RIF development. In addition, a number of recent studies have shown that single nucleotide polymorphisms (SNPs) or mutations within the miRNA sequence can lead to altered miRNA expression and/or maturation, and that SNPs in specific miRNAs contribute to the genetic predisposition for pregnancy loss (14).

To date, however, few studies have investigated the potential relationship between specific SNPs in miRNA machinery genes and the risk of RIF. Here, we investigated whether polymorphisms in the miRNA machinery genes *DROSHA, DICER1, RAN, XPO5,* and *DGCR8* are associated with increased risk of RIF in Korean women.

Materials and methods

Study participants. The study population consisted of women who experienced RIF (n=119) and normal controls (n=228). RIF was defined as the failure to achieve pregnancy after two completed fresh IVF-ET cycles, in subjects whose serum human chorionic gonadotrophin concentrations were <5 mIU/ml at 14 days after ET. Each transferred embryo was cleaved into more than 10 cells, and these were examined by the embryologist and judged to be of good quality by morphological assessment, according to the Gardner criteria (15), before transfer. Grade 1 embryos, with regular blastomeres and no cytoplasmic fragments, and grade 2 embryos, with regular blastomeres and minor cytoplasmic fragments, were considered to be good-quality embryos.

Both the male and female partners experiencing RIF were evaluated. Subjects who were diagnosed with RIF due to anatomic, chromosomal, hormonal, infectious, autoimmune, or thrombotic causes were excluded from the study group. Anatomical abnormalities were evaluated using several imaging modalities, including sonography, hysterosalpingogram, hysteroscopy, computerized tomography, and magnetic resonance imaging. Karyotyping was conducted using standard protocols. Hormonal causes for RIF, including hyperprolactinemia, luteal insufficiency, and thyroid disease, were excluded by measuring prolactin, thyroid-stimulating hormone (TSH), free T4, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone levels in peripheral blood. Lupus anticoagulant and anti-cardiolipin antibodies were measured to identify the autoimmune diseases. Thrombotic disorders, defined as thrombophilia, were evaluated by protein C and protein S deficiencies, and by the presence of anti-α2 glycoprotein antibodies. Semen analysis, karyotyping, and hormonal assays, including measurements of estradiol, testosterone, FSH, and LH, were performed for male partners. Among the initial 152 patients who were enrolled for the study, 33 subjects who had Müllerian anomaly, hypothyroidism, chromosomal abnormality, or antiphospholipid syndrome were excluded from the patient group, resulting in 119 RIF patients for the study.

Women in the control group were recruited from CHA Bundang Medical Center. Enrollment criteria for controls included regular menstrual cycles, normal karyotype (46, XX), a history of at least one naturally conceived pregnancy, and no history of pregnancy loss. Data were collected identically for both groups. Furthermore, this study was approved by the Institutional Review Board of CHA Bundang Medical Center (reference no. PBC09-120) and all participants provided written informed consent. All study protocols followed the recommendations of the Declaration of Helsinki.

Analysis of miRNA biogenesis gene polymorphisms. Peripheral blood samples for genotyping were collected from all subjects, using an anticoagulant. Genomic DNA was extracted from blood samples using the G-DEX IIb Genomic DNA Extraction Kit-BLOOD (iNtRON Biotechnology, Seongnam, South Korea). Nucleotide sequence variations in miRNA processing genes were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. Restriction enzyme digestion was carried out using the following enzymes (New England BioLabs) and conditions: *Nla*III (*DROSHA, rs10719*), *Ban*I (*DICER1*, rs3742330), *Bsl*I (*RAN*, rs14035), with incubation at 55˚C for 16 h, and *Eco*RI (*XPO5*, rs11077) and *Nsi*I (*DGCR8*, rs417309), with incubation at 37˚C for 16 h. Genotypes determined by RFLP analysis were confirmed by two independent investigators, and then checked again by sequencing 10% of the samples. For *RAN* rs14035 genotyping of the control group, and for *XPO5* rs11077 genotyping of RIF patients, genotypes were confirmed three times to rule out possible errors attributed to violation of the Hardy-Weinberg equilibrium (HWE).

Hormone assays. Blood samples were collected by venipuncture on day 2 or 3 of the menstrual cycle for the measurement of FSH, LH, E2, TSH, and prolactin levels. Serum was prepared as previously described (16), and hormone levels were determined using either radioimmunoassays (E2, TSH, and prolactin; Beckman Coulter), or enzyme immunoassays (FSH and LH; Siemens AG), according to the manufacturer protocols.

Immune cell assays. Immune cell measurements were performed by flow cytometry and analyzed using CellQuest

Table I. Baseline characteristics of controls and RIF patients.

Characteristic	Controls (n=210), mean ± SD	RIF patients (n=119), mean ± SD	P-value[a]
Age (years)	34.06±5.66	34.24±3.35	0.760
Platelet ($10^3/\mu l$)	239.47±66.35	238.46±63.65	0.919
PT (sec)	11.61±3.32	11.42±0.62	0.001[b]
aPTT (sec)	33.45±3.65	30.93±3.41	<0.001
FSH (mIU/ml)	8.14±2.86	8.76±4.50	0.662[b]
LH (mIU/ml)	3.26±1.78	4.75±2.33	<0.001[b]
Estradiol-2 (pg/ml)	26.17±14.83	34.20±23.25	0.001[b]
Body mass index (kg/m^2)	21.78±3.49	20.89±2.55	0.276[b]
Hematocrit (%)	35.95±4.19	-	-
Folate (nmol/l)	-	15.63±11.29	-
Homocysteine (umol/l)	-	6.56±1.34	-
BUN (mg/dl)	-	10.03±2.86	-
Creatinine (mg/dl)	-	0.78±0.10	-
Uric acid (mg/dl)	-	3.93±0.96	-
Total cholesterol (mg/dl)	-	192.93±42.77	-
White blood cell ($10^3/\mu l$)	-	7.22±2.85	-
TSH (uIU/ml)	-	2.26±1.44	-
Prolactin (ng/ml)	-	12.64±6.18	-
Hemoglobin (g/dl)	-	12.57±1.40	-
CD19+ B cell (%)	-	66.27±11.23	-
CD3+ pan T (%)	-	33.83±8.65	-
CD4+ helper T (%)	-	29.11±7.88	-
CD56+ NK cell (%)	-	11.01±4.44	-
CD8+ T (%)	-	20.00±9.49	-

[a]P-values were calculated by two-sided t-test for continuous variables and Fisher's exact test for categorical variables. [b]P-values were calculated by Mann-Whitney-test for continuous variables. RIF, recurrent implantation failure; SD, standard deviation; PT, prothrombin time; aPTT, activated partial thromboplastin time; BUN, blood urea nitrogen; TSH, thyroid stimulating hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

software (BD FACSCalibur; BD Biosciences), as previously described (17). Fluorescently labeled [fluorescein isothiocyanate, phycoerythrin (PE), peridinin chlorophyll protein, and allophycocyanin] monoclonal antibodies specific for CD3, CD4, CD8, CD19, and CD56 were obtained from BD Biosciences. Anti-NKG2A-PE antibodies were obtained from Immunotech (Beckman Coulter). These experiments were performed in the department of Diagnostic Laboratory Medicine, CHA Bundang Medical Center.

Statistical analysis. To compare baseline characteristics between RIF patients and controls, we used Chi-square tests to analyze categorical data, and the unpaired-samples Student's t-test to analyze continuous data. Also, we used analysis of variance (ANOVA) followed by Tukey's test to analyze continuous data. Genetic frequencies of each polymorphism in RIF patients and controls were compared using multivariate logistic regression. Allele frequencies were calculated to identify deviations from HWE using P=0.05 as a threshold. Odds ratios (ORs), adjusted odds ratios (AOR), and 95% confidence intervals (CIs) were used to measure the strength of the association between *DICER1, DROSHA, RAN, XPO5,* and *DGCR8* polymorphisms and risk of RIF with

GraphPad Prism 4.0 (GraphPad Software Inc.) and StatsDirect software, version 2.4.4 (StatsDirect Ltd.) (18). Associations between polymorphisms and RIF incidence were calculated using AORs and 95% CIs from logistic regression, adjusted for age. Two-tailed P-values <0.05 were considered statistically significant. A false discovery rate (FDR) correction was performed to adjust for multiple comparisons and associations with FDR-P-values <0.05 were considered statistically significant (19). Gene-gene interactions among SNP loci were analyzed using multifactor dimensionality reduction (MDR), with MDR, version 2.0 (www.epistasis.org) (20). We determined the best multilocus combinations based on MDR identification of the most significant models, using the optimal maximized cross-validation values. HAPSTAT, version 3.0 (www.bios.unc.edu/~lin/hapstat/), was used to estimate which allele combination frequencies, determined by MDR analysis, are predicted to have strong synergistic effects (21).

Results

Genotype frequencies of miRNA machinery gene polymorphisms. The demographic characteristics and clinical variables of RIF patients and control subjects are presented in Table I.

Table II. Genotype frequency of miRNA machinery gene polymorphisms between RIF patients and control groups.

Genotypes	Controls, n (%) (n=210)	RIF patients, n (%) (n=119)	AOR (95% CI)[a]	P-value
DICER rs3742330A>G				
AA	65 (31.0)	40 (33.6)	1.000 (reference)	
AG	114 (54.3)	60 (50.4)	0.856 (0.517-1.417)	0.546
GG	31 (14.8)	19 (16.0)	0.991 (0.493-1.992)	0.980
Dominant (AA vs. AG+GG)			0.890 (0.550-1.440)	0.634
Recessive (AA+AG vs. GG)			0.752 (0.275-0.000)	0.752
HWE *P*	0.096	0.656		
DROSHA rs10719T>C				
TT	95 (45.2)	68 (57.1)	1.000 (reference)	
TC	99 (47.1)	39 (32.8)	0.550 (0.339-0.893)	0.016
CC	16 (7.6)	12 (10.1)	1.062 (0.472-2.390)	0.884
Dominant (TT vs. TC+CC)			0.620 (0.394-0.976)	0.039
Recessive (TT+TC vs. CC)			1.365 (0.623-2.994)	0.437
HWE *P*	0.154	0.085		
RAN3'UTR rs14035C>T				
CC	112 (53.3)	77 (64.7)	1.000 (reference)	
CT	89 (42.4)	37 (31.1)	0.590 (0.363-0.958)	0.033
TT	9 (4.3)	5 (4.2)	0.759 (0.239-2.417)	0.641
Dominant (CC vs. CT+TT)			0.616 (0.386-0.982)	0.042
Recessive (CC+CT vs. TT)			0.976 (0.319-2.984)	0.965
HWE *P*	0.092	0.835		
XPO5 rs11077A>C				
AA	176 (83.8)	99 (83.2)	1.000 (reference)	
AC	32 (15.2)	18 (15.1)	0.983 (0.523-1.846)	0.957
CC	2 (1.0)	2 (1.7)	1.529 (0.202-11.601)	0.681
Dominant (AA vs. AC+CC)			1.036 (0.564-1.903)	0.910
Recessive (AA+AC vs. CC)			1.726 (0.236-12.618)	0.591
HWE *P*	0.687	0.283		
DGCR8 rs417309G>A				
GG	197 (93.8)	110 (92.4)	1.000 (reference)	
GA	13 (6.2)	9 (7.6)	1.237 (0.513-2.988)	0.636
AA	0 (0.0)	0 (0.0)	NA	NA
Dominant (GG vs. GA+AA)			1.237 (0.513-2.988)	0.636
Recessive (GG+GA vs. AA)			NA	NA
HWE *P*	0.643	0.668		

[a]Adjusted by age. RIF, recurrent implantation failure; AOR, adjusted odds ratio; HWE, Hardy-Weinberg equilibrium; 95% CI, 95% confidence interval; NA, not applicable.

The genotype and allele frequencies for the five miRNA machinery genes in RIF patients and unaffected controls are shown in Table II. All genes that were analyzed contained polymorphisms and the genotypic distributions in both groups were in HWE. We found that the *DROSHA* rs10719T>C and *RAN* rs14035C>T 3'UTR polymorphisms were negatively associated with the prevalence of RIF. Further, the frequencies of the TC genotype of *DROSHA* rs10719 and that of CT genotype of *RAN* rs14035 were lower in the RIF group than in control group (AOR=0.550, 95% CI, 0.339-0.893, P=0.016; AOR=0.590, 95% CI, 0.363-0.958, P=0.033, respectively). Similarly, the combined *RAN* rs14035 CT+TT and combined *DROSHA*

rs10719 TC+CC genotypes were associated with decreased prevalence of RIF (AOR=0.616, 95% CI, 0.386-0.982, P=0.042; AOR=0.620, 95% CI, 0.394-0.976, *P*=0.039, respectively). For the *DICER1* rs3742330A>G, *XPO5* rs11077A>C, and *DGCR8* rs417309G>A genotypes, there were no statistically significant differences between the RIF group and controls (Table SI).

Allele combination analysis. An allele-allele combination-based analysis of the five polymorphisms of the miRNA machinery genes (*DICER1, DROSHA, RAN, XPO5, DGCR8*) was conducted to evaluate whether polymorphic site interactions exert synergistic effects on RIF risk (Table III). Among

Table III. Allele combination frequencies for the miRNA machinery genes polymorphisms in RIF patients and controls.

Allele combinations	Controls, n (%) (2n=420)	RIF patients, n (%) (2n=238)	OR (95% CI)	P-value	FDR P-value
DICER1 A>G/DROSHA T>C/RAN 3'UTR C>T/XPO5 A>C/DGCR8 G>A					
A-T-C-A-G	112 (26.8)	83 (34.8)	1.000 (reference)		
A-T-C-C-G	21 (5.0)	5 (2.1)	0.321 (0.116-0.888)	0.022	0.095
A-C-T-A-G	26 (6.3)	4 (1.6)	0.208 (0.070-0.618)	0.002	0.022
G-T-C-A-G	81 (19.2)	37 (15.7)	0.616 (0.381-0.998)	0.048	0.168
G-T-C-C-G	0 (0.0)	10 (4.3)	28.290 (1.634-490.000)	<0.001	0.021
G-T-T-A-G	49 (11.6)	18 (7.7)	0.496 (0.269-0.913)	0.023	0.095
G-C-T-A-G	0 (0.0)	6 (2.4)	17.510 (0.972-315.500)	0.007	0.048
DICER1 A>G/DROSHA T>C/RAN 3'UTR C>T/XPO5 A>C					
A-T-C-A	104 (24.8)	84 (35.3)	1.000 (reference)		
A-T-C-C	24 (5.7)	7 (3.1)	0.361 (0.148-0.879)	0.021	0.062
A-C-T-A	30 (7.1)	4 (1.7)	0.165 (0.056-0.487)	<0.001	0.007
G-T-C-A	91 (21.6)	40 (16.8)	0.544 (0.340-0.871)	0.011	0.047
G-T-C-C	0 (0.0)	10 (4.1)	25.970 (1.499-450.000)	<0.001	0.007
G-T-T-A	46 (11.0)	19 (8.0)	0.511 (0.279-0.938)	0.029	0.062
G-C-T-A	1 (0.2)	7 (3.2)	8.667 (1.045-71.880)	0.026	0.062
DICER1 A>G/DROSHA T>C/RAN 3'UTR C>T/DGCR8 G>A					
A-T-C-G	133 (31.6)	88 (37.0)	1.000 (reference)		
A-C-T-G	30 (7.1)	6 (2.5)	0.302 (0.121-0.756)	0.008	0.072
G-C-T-G	0 (0.0)	5 (2.2)	16.590 (0.906-304.100)	0.011	0.072
DICER1 A>G/RAN 3'UTR C>T/XPO5 A>C/DGCR8 G>A					
A-C-A-G	167 (39.7)	107 (45.2)	1.000 (reference)		
G-C-C-G	5 (1.3)	11 (4.8)	3.434 (1.160-10.160)	0.019	0.262
DICER1 A>G/DROSHA T>C/RAN 3'UTR C>T					
A-T-C	130 (30.9)	90 (38.0)	1.000 (reference)		
A-C-T	34 (8.1)	6 (2.7)	0.255 (0.103-0.633)	0.002	0.008
G-C-T	0 (0.0)	7 (2.9)	21.630 (1.219-383.800)	0.002	0.008
DICER1 A>G/DROSHA T>C/XPO5 A>C					
A-T-A	127 (30.2)	99 (41.5)	1.000 (reference)		
A-T-C	26 (6.2)	7 (2.8)	0.345 (0.144-0.829)	0.014	0.024
A-C-A	87 (20.6)	31 (13.0)	0.457 (0.281-0.744)	0.002	0.005
G-T-A	137 (32.5)	59 (24.9)	0.553 (0.369-0.827)	0.004	0.009
G-T-C	0 (0.0)	10 (4.3)	26.910 (1.557-465.100)	<0.001	0.005
DICER1 A>G/DROSHA T>C/DGCR8 G>A					
A-T-G	152 (36.1)	103 (43.2)	1.000 (reference)		
A-C-G	85 (20.2)	34 (14.5)	0.590 (0.369-0.944)	0.027	0.190
DICER1 A>G/RAN 3'UTR C>T/XPO5 A>C					
A-C-A	167 (39.7)	109 (45.7)	1.000 (reference)		
A-T-C	8 (1.8)	0 (0.1)	0.090 (0.005-1.576)	0.025	0.178
DICER1 A>G/XPO5 A>C/DGCR8 G>A					
A-A-G	212 (50.4)	127 (53.5)	1.000 (reference)		
G-C-G	5 (1.2)	11 (4.6)	3.672 (1.247-10.810)	0.012	0.085

Table III. Continued.

Allele combinations	Controls, n (%) (2n=420)	RIF patients, n (%) (2n=238)	OR (95% CI)	P-value	FDR P-value
DICER1 A>G/DROSHA T>C					
A-T	154 (36.8)	106 (44.4)	1.000 (reference)		
A-C	90 (21.3)	34 (14.5)	0.549 (0.345-0.875)	0.011	0.033
DROSHA T>C/RAN 3'UTR C>T					
T-C	222 (52.9)	141 (59.4)	1.000 (reference)		
C-T	40 (9.6)	13 (5.6)	0.512 (0.264-0.991)	0.044	0.131
RAN 3'UTR C>T/XPO5 A>C					
C-A	286 (68.0)	169 (71.0)	1.000 (reference)		
T-C	9 (2.1)	0 (0.0)	0.089 (0.005-1.539)	0.030	0.089

Exclude criteria, P-value >0.05. RIF, recurrent implantation failure; 95% CI, 95% confidence interval; FDR, false discovery rate.
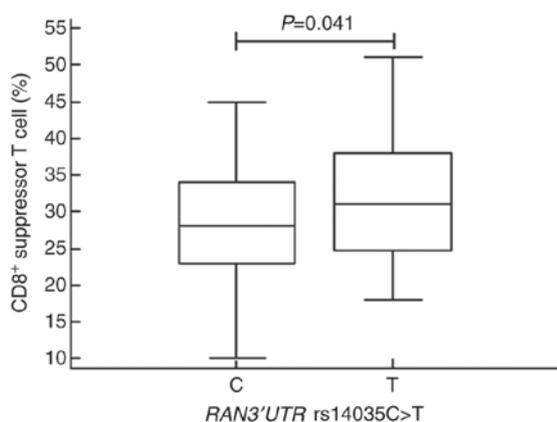
Figure 1. Proportions of CD8$^+$ T cells in subjects with the *RAN* 3'UTR C and T alleles. Different proportions of CD8$^+$ T cells (P=0.041) were detected in subjects with the *RAN* 3'UTR C (mean ± SD, 28.50±7.58) and T alleles (mean ± SD, 31.39±8.55). UTR, untranslated region; RAN, Ras-related nuclear protein.
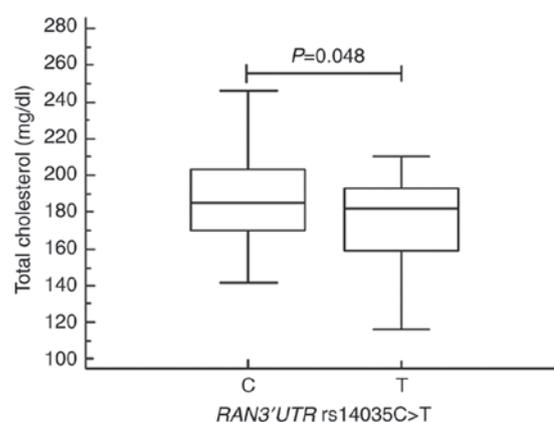
Figure 2. Total cholesterol levels in subjects with the *RAN* 3'UTR C and T alleles. Total cholesterol levels were significantly different (P=0.048) in subjects with the *RAN* 3'UTR C (mean ± SD, 196.68±45.01) and T alleles (mean ± SD, 176.33±23.89). UTR, untranslated region; RAN, Ras-related nuclear protein.

the models of five polymorphic loci, one allele combination (A-C-T-A-G) showed synergistic effects on reduced RIF risk (OR=0.208, 95% CI, 0.070-0.618, FDR-P=0.022), whereas two other allele combinations (G-T-C-C-G and G-C-T-A-G) were associated with increased risk of RIF (OR=28.290, 95% CI, 1.634-490.000, FDR-P=0.021; OR=17.510, 95% CI, 0.972-315.500, FDR-P=0.048, respectively), when the reference combination was A-T-C-A-G. However, it was difficult to make a conclusion with five allele combinations of G-T-C-C-G and G-C-T-A-G due to the wide range of CIs. Three-allele combination analysis indicated that *DICER1/DROSHA/RAN* A-C-T (OR=0.255, 95% CI, 0.103-0.633, FDR-P=0.008), as well as the *DICER1/DROSHA/XPO5* A-T-C, A-C-A, and G-T-A genotypes were associated with decreased RIF risk (OR=0.345, 95% CI, 0.144-0.829, FDR-*P*=0.024; OR=0.457, 95% CI, 0.281-0.744, FDR-*P*=0.005; and OR=0.553, 95% CI, 0.369-0.827, FDR-*P*=0.009, respectively). Among the models with two loci, the *DICER1/DROSHA* A-C genotype was associated with a reduced relative risk of RIF (OR=0.549, 95% CI, 0.345-0.875, FDR-P=0.033). These associations also

remained statistically significant after controlling for FDR. In addition, we performed combined genotype analysis with the miRNA machinery gene polymorphisms (Table IV) and found that the *DROSHA* TC and *RAN* CT combination decreased the risk of RIF. The combination of these two genotypes showed the lowest AOR with statistical significance in RIF risk (AOR=0.314, 95% CI, 0.147-0.673, FDR-P=0.023).

Clinical variables and miRNA machinery gene polymorphisms. We next stratified the mean values for our clinical variables according to miRNA machinery gene polymorphism status using one-way ANOVA (Table SII). From this analysis, we found that the mean proportions of CD8$^+$ T cells and the mean total cholesterol levels were significantly different in subjects with the *RAN* genotypes (P=0.041 and P=0.048, respectively). Specifically, the proportion of the CD8$^+$ T cells was lower in those with the *RAN* C allele [mean ± standard deviation (SD), 28.50±7.58) than in subjects with the T allele (mean ± SD, 31.39±8.55, P=0.041; Fig. 1). Further, patients with the *RAN* C allele contained a significantly higher level

Table IV. Combined genotype analysis for the miRNA machinery gene polymorphisms in RIF patients and controls.

Genotype combinations	Controls, n (%) (n=210)	RIF patients, n (%) (n=119)	AOR (95% CI)[a]	P-value	FDR P-value
DICER1 A>G/DROSHA T>C					
AA/TT	26 (12.4)	24 (20.2)	1.000 (reference)		
AA/TC	31 (14.8)	13 (10.9)	0.457 (0.194-1.081)	0.075	0.299
AG/TC	56 (26.7)	19 (16.0)	0.358 (0.163-0.786)	0.011	0.084
DICER1 A>G/RAN 3'UTR C>T					
AA/CC	38 (18.1)	28 (23.5)	1.000 (reference)		
AG/CT	51 (24.3)	19 (16.0)	0.495 (0.240-1.021)	0.057	0.454
DROSHA T>C/RAN3'UTR C>T					
TT/CC	54 (25.7)	45 (37.8)	1.000 (reference)		
TC/CT	44 (21.0)	12 (10.1)	0.314 (0.147-0.673)	0.003	0.023
DROSHA T>C/XPO5 A>C					
TT/AA	79 (37.6)	55 (46.2)	1.000 (reference)		
TC/AA	83 (39.5)	33 (27.7)	0.581 (0.341-0.991)	0.046	0.277
DROSHA T>C/DGCR8 G>A					
TT/GG	90 (42.9)	62 (52.1)	1.000 (reference)		
TC/GG	93 (44.3)	37 (31.1)	0.577 (0.350-0.952)	0.031	0.157
RAN 3'UTR C>T/XPO5 A>C					
CC/AA	94 (44.8)	61 (51.3)	1.000 (reference)		
CT/AC	14 (6.7)	4 (3.4)	0.333 (0.096-1.162)	0.085	0.437
RAN 3'UTR C>T/DGCR8 G>A					
CC/GG	108 (51.4)	72 (60.5)	1.000 (reference)		
CT/GG	81 (38.6)	33 (27.7)	0.597 (0.360-0.992)	0.046	0.232

[a]Adjusted by age. Exclusion criteria, P-value >0.1. RIF, recurrent implantation failure; 95% CI, 95% confidence interval; AOR, adjusted odds ratio; FDR, false discovery rate; N/A, not applicable; UTR, untranslated region; RAN, Ras-related nuclear protein.
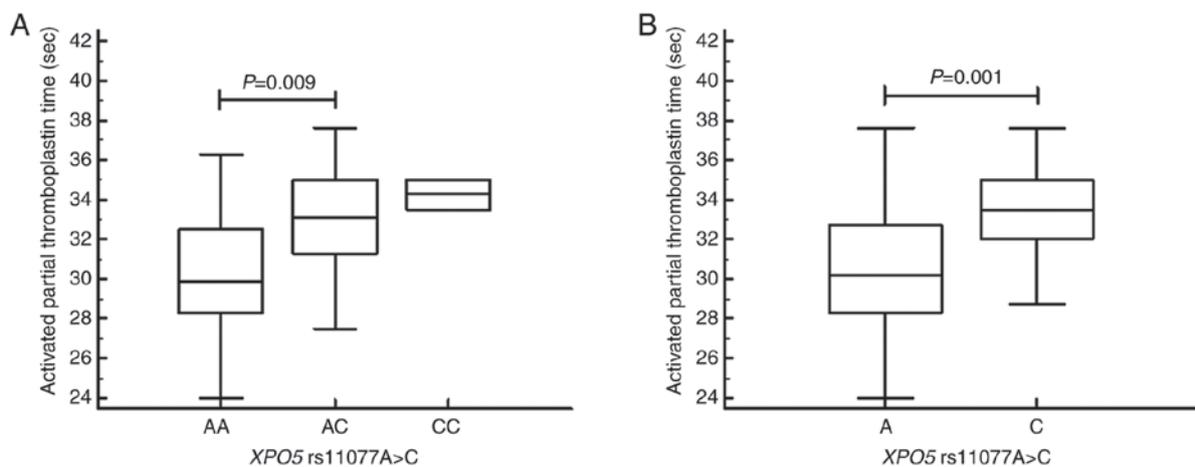
Figure 3. Activated partial thromboplastin time in subjects with *XPO5* A and C alleles. (A) Activated partial thromboplastin time was significantly different (P=0.009) in subjects with the *XPO5*AA (mean ± SD, 30.25±3.07) and AC (mean ± SD, 33.26±3.84) genotypes. (B) Activated partial thromboplastin time was significantly different (P=0.001) in subjects with the *XPO5*A (mean ± SD, 30.56±3.26) and C alleles (mean ± SD, 33.53±3.30). XPO5, exportin 5.

of total cholesterol (mean ± SD, 196.68±45.01) than those with the T allele (mean ± SD, 176.33±23.89, P=0.048; Fig. 2). In subjects with the various *DICER1* genotypes, the mean proportions of CD19+ B cells were significantly different; CD19+ B cells were higher in those with the GG vs. the AA genotype

(P=0.012, Fig. S1]. Further, the mean activated partial thromboplastin times (aPTT) and blood urea nitrogen (BUN) levels were significantly different (P=0.009 and P=0.037) in those with distinct *XPO5* genotypes and alleles (Figs. 3 and S2). That is, aPTT time was higher in those with the AC vs. the

AA genotype and in those with the C vs. the A allele, and BUN levels were higher in those with the AA vs. the CC genotype (Fig. 3A and B). Moreover, the optimal sample size was determined based on statistical power analysis (Tables SIII and SIV).

Discussion

In this study, we evaluated SNPs in five genes, *DROSHA, DICER1, RAN, XPO5,* and *DGCR8*, and their possible association with RIF. At present, the role that genetic variation in miRNA machinery genes may play in the development of RIF is not fully understood, although modifications in these genes significantly affect cellular transformation in various diseases (22-25) and carcinogenesis in several cancers (26-29). To the best of our knowledge, this is the first report to evaluate the association between miRNA machinery gene polymorphisms and RIF. We found that the *DROSHA* rs10719T>C and *RAN* rs14035C>T 3'UTR polymorphisms are significantly associated with decreased prevalence of RIF in Korean women. Further, the *DROSHA* rs10719 TC and *RAN* rs14035 CT genotype frequencies were found to be lower in the RIF group than in the control group. This observation suggests that each of these confer protective effects against RIF occurrence. We also examined potential interactive effects of different genetic polymorphisms on RIF using genotype-based MDR analysis, which can detect gene-gene interactions, regardless of chromosomal location (20). An allele-allele combination-based analysis of the five polymorphisms indicated that there is a gene-gene interaction between the *DROSHA* rs10719 and the *RAN* rs14035 polymorphisms. In particular, five-allele combination analysis showed that the *DICER1/DROSHA/RAN/XPO5/DGCR8* A-C-T-A-G combination is associated with decreased RIF risk, when the reference combination is A-T-C-A-G, which indicates that both the *DROSHA* and *RAN* polymorphisms exert synergistic effects on reducing RIF risk. Four, three, and two-allele combination analysis revealed similar results. In our genotype combination analysis, the *DROSHA* TC genotype showed a highly significant negative association with RIF prevalence when combined with the *RAN* CT genotype, which implies that defective *DROSHA* function, arising due to genetic polymorphisms, might be intensified by *RAN* polymorphisms.

DROSHA is a nuclear RNase III enzyme responsible for cleaving pri-miRNAs into pre-miRNAs, and thus is essential for miRNA biogenesis and the maturation. DROSHA also recognizes and directly cleaves many protein-coding messenger RNAs with secondary stem-loop structures (7). In addition to DROSHA, miRNA biogenesis requires the RNase III enzyme, DICER1. Numerous studies have identified roles for DROSHA and DICER1 in various diseases (22-25) and in a variety of cancers (27-31). For example, Kim *et al* (22) found that the *DICER* rs3742330 A>G and *DROSHA* rs10719 T>C polymorphisms are more common in ischemic stroke patients. Additionally, Saeki *et al* (23) reported an association between the development of Graves' disease and the *DICER* rs1057035 TT and *DROSHA* rs644236 CC genotypes. Zhang *et al* (24) further showed that the *DROSHA* rs10719 T>C polymorphism promotes the development of primary hypertension by disrupting the interaction between

miR-27b and *DROSHA*, and both the *DROSHA* rs10719 TC and CC genotypes were found to be associated with increased susceptibility to preeclampsia (25).

In reproductive disease, however, the expression of *DROSHA* and its role remain unclear. Zhang *et al* (32) analyzed *Drosha* expression patterns in the mouse uterus during early pregnancy and concluded that this gene is involved in stromal decidualization and may participate in the process of embryo implantation in mice. *Drosha* expression in the endometrium was gradually up-regulated around the implantation window, reached a peak on the sixth day of pregnancy, and was reduced thereafter. However, few reports have evaluated the clinical significance of the *DROSHA*, *DICER,* and *RAN* SNPs in human reproduction. A recent study by Jung *et al* (33) revealed that the *RAN* rs14035 CC genotype and the combined *DICER* rs3742330/*DROSHA* rs10719 GG/TC+CC, *DICER* rs3742330/*RAN* rs14035 GG/CC, and *DICER* rs3742330/*XPO5* rs11077 GG/AC+CC genotypes were significantly associated with an increased risk of recurrent pregnancy loss (RPL) in Korean women. Moreover, they showed that the *RAN* rs14035 CT genotype and combined *DICER* rs3742330/*RAN* rs14035 AA+AG/CT+TT, *DROSHA* rs10719/*RAN* rs14035 TC+CC/CT+TT, and *RAN* rs14035/*XPO5* rs11077 CT+TT/AA genotypes were associated with a reduced risk of RPL. Fu *et al* (34) further reported that the *DICER* rs3742330/*DROSHA* rs10719 GG/TC+TT combinations were associated with an increased risk of RPL in the Chinese population. Additionally, down-regulation of *DROSHA* has been associated with higher histological grades in endometrial cancer (28) and decreased median survival of ovarian cancer patients (29), suggesting a role for DROSHA in reproductive cancers, as well.

Here, we found that the combined *RAN* rs14035 CT+TT genotype was associated with decreased RIF risk, and that *RAN* rs14035 CT heterozygotes have a significantly decreased relative risk of RIF, as compared to wild type homozygotes. RAN, a member of the Ras superfamily of GTPases, is essential for the transport of pre-miRNAs from the nucleus to the cytoplasm through the nuclear pore complex (35). RAN also plays key roles in assembly of the mitotic spindle and nuclear envelope during mitosis. When RAN-GTP is depleted, pre-miRNA export is greatly reduced, indicating that disruption of the RAN pathway perturbs the miRNA transport processes (36). Therefore, it is possible that RAN mutations mediate pathologic changes in miRNA transport and expression. RAN is also a well-known downstream modulator of osteopontin-induced malignant transformation of cells, which mediates cancer cell invasion and metastasis (37). Overexpression of RAN stimulates cancer cell differentiation, invasion, and ultimately metastasis in colorectal cancer (38). Consistent with these data, Yuen *et al* (39) demonstrated that knockdown of *RAN* suppressed adhesion, migration, and invasion of cancer cells in several breast and lung cancer cell lines.

To our knowledge, there are no published studies describing the effects of these SNPs specifically on RIF. Critically, we note that the pathophysiologies of RIF and RPL are distinct, although their respective etiologies have not been clearly elucidated. RPL is a disease that is distinct from infertility, whereas RIF occurs in a specific group of infertile patients

undergoing IVF (40). In our study, both the *DROSHA* rs10719 TC and *RAN* rs14035 CT genotypes were associated with the prevalence of RIF. However, Jung *et al* (33) did not find an association between the *DROSHA* rs10719 polymorphism and RPL risk. Furthermore, four miRNA polymorphisms of the same patient and control groups in our previous study were not associated with the prevalence of RIF in genotype analysis but significantly associated with the RIF risk in allele combination analysis and interaction analysis (41). The results of combined genotype analyses for RPL and RIF were also different, which further implies distinct etiologies for these conditions. Interestingly, however, the *RAN* rs14035 CT genotype was related to a reduced risk of both RPL and RIF, suggesting that these conditions may also share some underlying mechanistic features, as well.

During early implantation, the endometrium undergoes dynamic cellular processes, including DNA synthesis and angiogenesis, which are required for cell proliferation and decidualization, in order to become receptive to the embryo (42). We hypothesize that dysregulation of miRNA machinery genes may locally affect angiogenesis and decidualization in the endometrium during implantation and pregnancy maintenance in early pregnancy. DROSHA may be particularly important for implantation, as this enzyme is essential for posttranscriptional miRNA processing. In both the individual and combined genotyping analysis of our study, the *DROSHA* rs10719 TC genotype was inversely associated with the RIF prevalence. In the combination analysis, *DROSHA* TC genotype combined with the *RAN* CT genotype showed the most significant negative association with RIF development. However, it remains unclear how the polymorphisms in these genes may influence one another during implantation.

A critical role for miRNAs in the differentiation of human T cells is becoming increasing evident (43). For example, it has been shown that miRNAs regulate thymic development of both CD4+ and CD8+ T cells and are further involved in the differentiation of mature T cells (44). *DICER1* is also involved in the control of CD8+ T cell activation, proliferation, migration, and accumulation (44,45). Experiments in mice with a conditional deletion of *Dicer1* in mature CD8+ T cells revealed a dramatically reduced CD8+ effector T cell response in mutant animals, suggesting an essential function for miRNAs in the regulation of T cells (45). In our study, we found that subjects with the *RAN* rs14035 T allele had a significantly higher proportion of CD8+ T cells than those with the *RAN* rs14035 C allele (P=0.041). Notably, this *RAN* polymorphism was associated with decreased risk of RIF in our population. Several observational studies have suggested that T cells and NK cells may be involved in RIF (46,47). For example, Yang *et al* (46) found that women with RIF have increased T cell activation in peripheral blood lymphocytes. Our results also suggest a role for T cells in RIF, although we found that a *RAN* polymorphism associated with a higher proportion of CD8+ T cells is associated with decreased risk of RIF. In contrast, Yin *et al* (47) detected no significant difference in the proportions of peripheral CD8+ T cells in women with RIF and in healthy controls. A potential explanation for these conflicting results is that RIF has various unknown etiologies, and the role of immunologic factors in implantation failure is both complex and poorly understood (48). Thus, although our data suggest that CD8+ T cells are involved in implantation failure, the precise role that these cells play in RIF remains unclear. Conversely, we found no significant association between NK cells and the other miRNA machinery gene polymorphisms. In addition, the question of whether other miRNA machinery genes may affect the differentiation of T cells and the risk of RIF has not yet been elucidated and will require further investigation.

There are several limitations to this study. First, the association between miRNA machinery gene polymorphisms and RIF risk found in our investigation is not a causal relationship. Although our results suggest potential roles of these SNPs in the development of RIF, it is difficult to definitively conclude that miRNA machinery genes affect RIF. Therefore, further research will be needed to explore this possibility. Second, the mechanism underlying the role of these miRNA machinery gene polymorphisms and the development of RIF has not been demonstrated. Functional studies of which polymorphisms affect miRNA biogenesis and promote the RIF pathogenesis were not conducted, as they were beyond the scope of this study. Third, we recruited women with normal pregnancy history for the control group, rather than women with successful pregnancy after IVF-ET. Thus, with the current comparison, we cannot rule out the possibility that these SNPs are associated with infertility rather than with RIF, specifically. Lastly, miRNA expression in local tissue, such as endometrial tissue, in individuals with different SNP genotypes, was not examined. Altered expression of miRNAs could change endometrial expression of specific genes critical for embryo implantation (49,50). Lastly, the sample size of our RIF group was not big enough to provide sufficient statistical power for demonstrating association between these miRNA SNPs and RIF. Thus, future studies with larger sample sizes will be required to confirm these results.

In conclusion, we have identified an association between the *DROSHA* rs10719 and *RAN* rs14035 3'UTR polymorphisms and the risk of RIF in Korean women. Our data demonstrate that the frequencies of the TC genotype of *DROSHA* rs10719 and that of the CT genotype of *RAN* rs14035 are lower in RIF patients than in controls, suggesting that these alleles may be associated with reduced risk of RIF. The underlying mechanisms by which these alleles affect RIF remain unknown and will be investigated in future studies. Regardless, our data suggest that these miRNA machinery gene polymorphisms could function as potential diagnostic markers to predict the RIF occurrence.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YL, EHA and NKK conceived and designed the experiments. YL, EHA, CSR, HJA, SHC, JHK and YRK performed the experiments. YL, EHA, CSR, JOK, YRK, WSL and NKK analyzed the data. YL, EHA and NKK wrote the paper. YL, EHA and NKK edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Institutional Review Board of CHA Bundang Medical Center reviewed and approved the current study on February 23, 2010 (reference no. PBC09-120), and written informed consent was obtained from all participants.

Patient consent for publication

All patients provided written informed consent and agreed to the publication of the present study.

Competing interestsin

The authors declare that they have no competing interests.

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