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GENETICS

Genetic polymorphisms of the cobalamin transport system are associated with idiopathic recurrent implantation failure

Han Sung Park[1] · Jung Oh Kim[1] · Hui Jeong An[1] · Chang Soo Ryu[1] · Eun Ju Ko[1] · Young Ran Kim[2] · Eun Hee Ahn[2] · Woo Sik Lee[3] · Ji Hyang Kim[2] · Nam Keun Kim[1]

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Abstract

Purpose Vitamin B12 (cobalamin, Cbl) plays a role in the recycling of folate, which is important in pregnancy. Transcobalamin II (TCN2) and transcobalamin receptor (TCblR) proteins are involved in the cellular uptake of Cbl. TCN2 binds Cbl in the plasma, and TCblR binds TCN2-Cbl at the cell surface. Therefore, we investigated the potential association between polymorphisms in Cbl transport proteins, TCN2 and TCblR, and recurrent implantation failure (RIF) susceptibility.

Methods The genotypes of *TCN2* 67A>G, *TCN2* 776C>G, and *TCblR* 1104C>T were determined for RIF patients and healthy controls using a polymerase chain reaction restriction fragment length polymorphism assay. Additionally, statistical analysis was performed to compare the genotype frequencies between RIF patients and controls.

Results The *TCN2* 67 polymorphism AG type was associated with RIF risk. Some allele combinations that contained the *TCN2* 67 polymorphism G allele were associated with increased RIF risk, whereas other allele combinations that contained the *TCblR* 1104 polymorphism T alleles were associated with decreased RIF risk. In genotype combination analysis, two combinations containing the *TCN2* 67 polymorphism AG type were associated with RIF risk.

Conclusion Our study showed that the polymorphisms of *TCN2* and *TCblR* are associated with RIF and are potential genetic predisposing factors for RIF among Korean women. Additionally, our findings support a potential role for *TCN2* and *TCblR* in RIF among Korean women. However, further studies are required to investigate the role of the polymorphisms in those proteins and RIF because the roles of the *TCN2* and *TCblR* polymorphisms in RIF are not clear.

Keywords Genetic association · Recurrent implantation failure (RIF) · Vitamin B12 (cobalamin, Cbl) · Transcobalamin II · Transcobalamin receptor

Introduction

Implantation is the first stage of pregnancy in which the embryo attaches to the luminal surface of the endometrium and invades into the deep layers of the endometrium. Recurrent implantation failure (RIF) is a symptom whereby the implanted embryo has undergone two or more repeated failures before being at a recognizable stage by ultrasonography [1]. Additionally, RIF may be defined as the failure to maintain a clinical pregnancy following three in vitro fertilization (IVF) embryo transfer (ET) cycles [2]. Several studies have reported various causes of RIF, including embryo, anatomic, endometrial, uterine, and immunological factors as well as thrombophilic conditions. However, the genetic mechanisms underlying RIF still remain unclear [3, 4].

Folate metabolism plays an important role in DNA synthesis, cell proliferation, embryogenesis, implantation, and

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✉ Ji Hyang Kim
bin0902@chamc.co.kr

✉ Nam Keun Kim
nkkim@cha.ac.kr

[1] Department of Biomedical Science, College of Life Science, CHA University, 355, Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do 13488, Republic of Korea

[2] Department of Obstetrics and Gynecology, CHA Bundang Medical Center, CHA University, 11, Yatap-ro 65beon-gil, Bundang-gu, Seongnam-si, Gyeonggi-do 13496, Republic of Korea

[3] Fertility Center of CHA Gangnam Medical Center, CHA University, 566, Nonhyeon-ro, Gangnam-gu, Seoul 06135, Republic of Korea

ovarian function [5]. A folate deficiency can affect highly proliferative cells, such as neural tube cells, in the developing fetus, resulting in an enhanced risk of neural tube defects (NTDs) [6]. Folate levels are usually decreased by poor dietary intake or malabsorption [7], and folate supplementation is important in pregnant women. Folate supplementation increases plasma folate levels, but this increase is not associated with pregnancy outcomes in infertile women getting IVF treatment [8].

Vitamin B12 (cobalamin, Cbl) is essential for mammals and plays an important role in folate recycling, one-carbon metabolism, and DNA synthesis [9]. Cbl acts as a cofactor for enzymes in two forms, methyl-Cbl for methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase, *MTR*) and 50-deoxyadenosyl-Cbl (Ado-Cbl) for methylmalonyl-CoA mutase. Transport of Cbl is performed by Cbl-transporting proteins, including intrinsic factor, transcobalamin (TC), and haptocorrin (HC). Deficiency in Cbl is common in pregnant women and is associated with hyperhomocysteinemia, which is a risk factor for NTDs and recurrent spontaneous abortion (RSA) [10–16]. Supplementation can restore Cbl to normal levels [17–20].

Transcobalamin proteins are a member of the Cbl-binding protein family and are classified as transcobalamin I (TCN1) and transcobalamin II (TCN2) [21]. TCN2 and its corresponding receptor, TCblR (transcobalamin II receptor, CD320), play an important role in the absorption of Cbl into the cell. TCN2 is a plasma protein which binds Cbl, and this TCN2-Cbl combination specifically binds with the cell surface receptor TCblR [5]. Cellular deficiency of Cbl caused by TCN2 can occur via three mechanisms: a lack of TCN2 to bind Cbl, the absence of immunoreactive TCN2, and non-functional TCN2 binding to Cbl [22]. TCN2 is reported to be associated with various diseases, including omphaloceles [23], Alzheimer's disease [24], and stroke [25]. Additionally, we have reported on the association of *TCN2* and *TCblR* polymorphisms with RSA in a previous study [10]. In this study, we investigated whether *TCN2* and *TCblR* polymorphisms are associated with RIF and whether these polymorphisms affect the levels of folate and homocysteine in Korean women.

Materials and methods

Study participants

Blood samples were collected from 116 patients with idiopathic RIF (mean age ± SD 34.10 ± 3.17 years) and 204 female control participants (mean age ± SD 32.84 ± 4.32 years). The participants were recruited from the Department of Obstetrics and Gynecology of CHA Bundang Medical Center (Seongnam, South Korea) between March 2010 and December 2012. RIF was defined as the failure to achieve pregnancy following two or more completed in vitro fertilization-embryo transfer (IVF-ET) cycles with one or two good quality embryos. Each transferred embryo was cleaved into more than 10 cells, and one of these cells was used for karyotyping. Fourteen days after ET, all RIF patients' serum human chorionic gonadotrophin (hCG) concentrations were less than 5 U/ml. All transferred embryos were examined by the embryologist prior to transfer and judged to be of good quality. The male and female partner of each couple experiencing RIF was evaluated. Subjects who were diagnosed with RIF because of anatomical, chromosomal, hormonal, infectious, autoimmune, or thrombotic causes were excluded from this study group. Anatomical abnormalities, including uterine fibroids, septate uterus, and intrauterine adhesion, were evaluated using hysteroscopy, computed tomography scan, magnetic resonance imaging, and hysterosalpingogram. Chromosomal abnormalities, including trisomy, monosomy, triploidy, and translocation, were evaluated using karyotyping. Karyotyping was conducted using standard protocols. Hormonal causes, including hyperprolactinaemia, thyroid disease, and luteal insufficiency, were evaluated using blood measurements. RIF patients with a shorter luteal phase than 10 days or a lower progesterone level than normal during luteal phase were excluded from the study. Moreover, to determine whether RIF patients have luteal insufficiency, follicle stimulating hormone (FSH), luteinizing hormone (LH), and progesterone levels were measured, and ultrasonography was performed. Additionally, estradiol (E2) levels were measured on 2–3 days after initiation of menstruation (basal) and on the day of hCG injection (inj. hCG), in RIF patients. Infectious causes, including *Ureaplasma urealyticum* and *Mycoplasma hominis* infections, were evaluated using bacteriological culture. Autoimmune causes were evaluated using lupus anticoagulant and anticardiolipin antibodies. Thrombotic causes were evaluated by detecting deficiencies of protein C, protein S, and anti-beta-2 glycoprotein.

Genetic analysis

Two *TCN2* SNPs (rs9606756, *TCN2* 67A>G; rs1801198, *TCN2* 776C>G) and one *TCblR* SNP (rs9426, *TCblR* 1104C>T) were selected using the human genome SNP database (dbSNP; http://www.ncbi.nlm.nih.gov/snp). DNA was extracted from the blood samples using the G-DEX blood extraction kit (iNtRON Biotechnology, Seongnam, Korea). A polymerase chain reaction (PCR)-restriction fragment length polymorphism assay was used for the genotyping of all participants [26]. The information on the PCR primers, restriction enzymes, and enzyme reaction conditions for SNP genotyping is presented in Supplement Table 1; the modified base in the primer is underlined. All PCR conditions, except for the annealing temperature for three SNPs, were the same. The initial denaturation at 95 °C for 5 min was

followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The annealing temperatures for *TCN2* 67A > G, *TCN2* 776C > G, and *TCblR* 1104C > T were 58 °C, 54 °C, and 60 °C, respectively.

Estimation of homocysteine, folic acid, total cholesterol, and uric acid levels

Blood samples were collected into anticoagulant-containing collection tubes, and the samples were centrifuged for 15 min at 1000×*g* to separate the plasma from the whole blood. The homocysteine level was determined using the IMx fluorescence polarizing immunoassay (Abbott Laboratories, Abbott Park, IL, USA), and the folic acid level was measured using a radioassay kit (ACS:180; Bayer, Tarrytown, NY, USA). The total cholesterol (T.chol) and uric acid levels were determined using commercially available enzymatic colorimetric tests (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis

The association between *TCN2* and *TCblR* polymorphisms and RIF risk was examined using odds ratios (ORs), adjusted odds ratios (AORs), and 95% confidence intervals (CIs). The data are presented as numbers and percentages (categorical variables). The statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA), MedCalc version 12.1.4 (MedCalc Software bvba, Mariakerke, Belgium) and StatsDirect (V2.6.6 www.statsdirect.com). Haplotype frequencies were estimated using the HAPSTAT program (V3.0, www.bios.unc.edu/~lin/hapstat/) for the polymorphisms determined to have strong synergistic effects. Additionally, statistical power analysis was performed on data with a significant *P* value using GPower program (V3.1, www.gpower.hhu.de) (Supplement Table 2).

Results

Study population

The clinical characteristics and profiles of RIF patients and normal controls are summarized in Table 1. The average age of the control and RIF groups was not significantly different. Additionally, we used the age of the participants for the AOR in other statistical analyses. The average number of live births and the average gestation period of control subjects were 1.70 ± 0.59 and 39.19 ± 1.61, respectively. Additionally, no RIF patients had live births.

Table 1 Clinical profiles between RIF patients and controls subjects

Characteristics	Controls (*n* = 197)	RIF (*n* = 110)	*P*
Age (years)	33.14 ± 4.07	33.76 ± 2.91	0.122
BMI (kg/m^2)	21.74 ± 3.37	21.04 ± 2.78	0.121
Live births (*N*)	1.71 ± 0.59	None	
Mean gestational age (weeks)	39.21 ± 1.60	None	
Homocysteine (μmol/L)	None	6.47 ± 1.31	
Folate (mg/mL)	None	15.22 ± 11.31	
Total cholesterol (mg/dL)	None	189.59 ± 45.92	
Uric acid (mg/dL)	None	3.94 ± 0.98	
CD56+ NK cells (%)	None	20.02 ± 9.65	
CD19 (B cell)	None	11.19 ± 4.50	
CD3 (pan T)	None	65.98 ± 11.43	
CD4 (helper T)	None	33.39 ± 8.41	
CD8 (suppressor T)	None	29.30 ± 7.99	
E2 (Basal)	None	35.50 ± 24.75	
E2 (Inj. hCG)	None	2457.63 ± 2443.99	
PLT (10^3/μL)	239.70 ± 63.30	232.86 ± 57.89	0.373
PT (s)	11.34 ± 2.99	11.08 ± 1.53	0.560
aPTT (s)	33.29 ± 3.62	29.32 ± 3.49	< 0.0001
BUN (mg/dL)	None	10.24 ± 2.87	
Creatinine (mg/dL)	None	0.78 ± 0.10	

RIF recurrent implantation failure, *BMI* body mass index, *E2 (Inj. hCG)* estradiol (after inject human chorionic gonadotrophin), *PLT* platelet, *PT* prothrombin time, *aPTT* activated partial thromboplastin time, *BUN* blood urea nitrogen

Genotype frequency

The *TCN2* 67A>G polymorphism was significantly associated with greater odds of RIF (AA vs. AG: AOR 4.732; 95% CI 1.220 to 18.356; $P = 0.025$) in Table 2. This tendency was maintained in two subgroups, the three or more IFs per patient group (AA vs. AG: AOR 4.413; 95% CI 1.103 to 17.660; $P = 0.036$) and the four or more IFs per patient group (AA vs. AG: AOR 5.340; 95% CI 1.278 to 22.325; $P = 0.022$). The *TCN2* 776C>G polymorphism and the *TCblR* 1104C>T polymorphism were not significantly different between controls and total RIF or patients with three or more IFs. However, the CT types (AOR 0.329; 95% CI 0.123 to 0.876; $P = 0.026$) and CT + TT types (AOR 0.363; 95% CI 0.147 to 0.901; $P = 0.029$) of the *TCblR* 1104C>T polymorphism were significantly different in the four or more IF patient group.

Allele combination

We performed allele combination analysis using the three SNPs of the *TCN2* gene and *TCblR* gene to determine whether any specific allele combinations were associated with RIF (Table 3). In three allele combinations, the A-C-T type (OR 0.195; 95% CI 0.058 to 0.661; $P = 0.004$) significantly decreased the odds ratio and G-G-C type (OR 11.850; 95% CI 1.437 to 97.690; $P = 0.006$) significantly increased the odds ratio when compared with the A-C-C types. In the two-allele combination analysis, the G-G (OR 7.519; 95% CI 1.568 to 36.040; $P = 0.005$) of *TCN2* 67A>G and 776C>G, the G-C (OR 4.101; 95% CI 1.048 to 16.040; $P = 0.043$) of *TCN2* 67A>G and *TCblR* 1104C>T, and the C-T (OR 0.263; 95% CI 0.089 to 0.776; $P = 0.009$) of *TCN2* 776C>G and *TCblR* 1104C>T were associated with RIF.

Genotype combination

We also performed genotype combination analysis using two genotypes of *TCN2* and *TCblR* polymorphisms to determine whether any specific genotype combinations were associated with RIF (Table 4). In the *TCN2* 67A>G and *TCblR* 1104C>T genotype combination, the AG/CC (OR 4.038; 95% CI 1.018 to 16.008; $P = 0.047$) types were associated with RIF.

Level of risk factors according to *TCN2* and *TCblR* polymorphisms

Various clinical parameters were measured in RIF women. The levels of homocysteine, platelets, and hemoglobin according to patient genotype of the *TCN2* and *TCblR* polymorphisms are presented in Table 5. The *TCN2* 776C>G and *TCblR* 1104C>T polymorphisms were associated with the levels of hemoglobin. Additionally, the *TCN2* 776C>G polymorphism demonstrated an association with homocysteine levels. However, the *TCN2* 67A>G polymorphism was not associated with any clinical parameters.

Discussion

Successful embryo implantation requires both a synchronous development and an interaction between the hatched blastocyst and the endometrium [27]. The causes of implantation failure are diverse, and many are due to maternal factors, including hormonal and metabolic disorders, infections, immunological factors, and uterine abnormalities [3, 4]. Some recent studies have also investigated the contribution of embryo-cumulus cells, cells surrounding the oocyte, to the implantation process [28]. However, RIF may still occur even if none of these factors are found to be the cause.

The balance of homocysteine and folate is an important factor in pregnancy [29]. Homocysteine is created during the methionine pathway, and it can then be converted back to methionine or converted to cysteine. When vitamin B6 is deficient, homocysteine is unable to convert to cysteine. It also cannot be converted back to methionine without Cbl. Therefore, when certain members of the vitamin B group are insufficient, homocysteine levels increase [30]. Homocysteine is an oxidant that can damage blood vessels and cause cardiovascular diseases such as stroke [31] and myocardial infarction [32]. Homocysteine induces embryonic defects in both the neural tube and the heart. It also increases the risk of growth retardation and somite development abnormalities in mouse and rat embryos [33]. Homocysteine may be involved in the pathogenesis of idiopathic RIF [34]. Therefore, a high maternal homocysteine level may affect the transferred IVF embryo development.

Deficiencies in folate and Cbl are fetal development risk factors for pathogenic conditions, including neural tube, limb, cardiac, and jaw defects [35–38]. Cbl, required for folate dependent homocysteine metabolism, is often deficient in pregnant women [39]. Cbl deficiency during gestation and lactation results in postnatal growth retardation and decreases respiratory activity with disturbed mitochondrial alignment [40]. A Cbl deficiency can also cause macrocytic anemia, which results in the production of fewer but larger red blood cells and decreases the amount of hemoglobin in the blood [41]. Folate is essential for fetal development, and it plays a role as a cofactor in many biological processes, including the transfer of a single carbon [42]. In addition, folate affects the metabolism of several amino acids, including those involved in the trans-methylation and trans-sulfuration pathways. Folate is also involved in homocysteine re-methylation, and a folate deficiency can increase the level of homocysteine [43]. When the levels of vitamin B6, Cbl, and folate are sufficient, the homocysteine level is decreased and the levels of methionine and cysteine are increased. Methionine is essential for cell proliferation, DNA synthesis,

Table 2 Genotype frequencies of *TCN2* and *TCblR* gene polymorphisms in RIF patients and control subjects

Genotypes	Controls ($n = 197$)	RIF ($n = 110$)	AOR (95% CI)	*P*	FDR-*P*	IF ≥ 3 ($n = 104$)	AOR (95% CI)	*P*	FDR-*P*	IF ≥ 4 ($n = 73$)	AOR (95% CI)	*P*	FDR-*P*
TCN2 67A>G													
AA	194 (98.5)	102 (92.7)	1.000 (reference)			92 (92.9)	1.000 (reference)			64 (91.4)	1.000 (reference)		
AG	3 (1.5)	8 (7.3)	4.732 (1.220–18.356)	0.025	0.075	7 (7.1)	4.413 (1.103–17.660)	0.036	0.108	6 (8.6)	5.340 (1.278–22.325)	0.022	0.039
GG	0 (0.0)	0 (0.0)	None	None		0 (0.0)	None	None		0 (0.0)	None	None	
Dominant (AA vs AG + GG)			4.732 (1.220–18.356)	0.025	0.075		4.413 (1.103–17.660)	0.036	0.108		5.340 (1.278–22.325)	0.022	0.044
Recessive (AA+AG vs GG)			None	None			None	None			None	None	
HWE-P	0.914	0.692											
TCN2 776C>G													
CC	52 (26.4)	27 (24.5)	1.000 (reference)			26 (26.3)	1.000 (reference)			18 (25.7)	1.000 (reference)		
CG	100 (50.8)	54 (49.1)	1.008 (0.566–1.792)	0.980	0.98	46 (46.5)	2.478 (0.281–21.883)	0.414	0.414	35 (50.0)	0.955 (0.488–1.868)	0.893	0.893
GG	45 (22.8)	29 (26.4)	1.237 (0.637–2.405)	0.530	0.582	27 (27.3)	1.190 (0.605–2.341)	0.614	0.651	17 (24.3)	1.092 (0.500–2.381)	0.826	0.834
Dominant (CC vs CG + GG)			1.073 (0.625–1.842)	0.798	0.798		0.968 (0.557–1.682)	0.907	0.907		0.994 (0.531–1.861)	0.984	0.984
Recessive (CC + CG vs GG)			1.170 (0.680–2.014)	0.571	0.629		1.215 (0.695–2.125)	0.495	0.692		1.032 (0.540–1.973)	0.923	0.925
HWE-P	0.817	0.851											
TCblR 1104C>T													
CC	157 (79.7)	96 (87.3)	1.000 (reference)			85 (85.9)	1.000 (reference)			64 (91.4)	1.000 (reference)		
CT	37 (18.8)	13 (11.8)	0.565 (0.286–1.119)	0.102	0.153	13 (13.1)	0.639 (0.321–1.269)	0.201	0.302	5 (7.1)	0.329 (0.123–0.876)	0.026	0.039
TT	3 (1.5)	1 (0.9)	0.527 (0.054–5.150)	0.582	0.582	1 (1.0)	0.591 (0.060–5.783)	0.651	0.651	1 (1.4)	0.784 (0.080–7.700)	0.834	0.834
Dominant (CC vs CT + TT)			0.562 (0.290–1.088)	0.087	0.131		0.634 (0.326–1.234)	0.180	0.270		0.363 (0.147–0.901)	0.029	0.044
Recessive (CC + CT vs TT)			0.571 (0.059–5.565)	0.629	0.629		0.630 (0.065–6.161)	0.692	0.692		0.896 (0.091–8.792)	0.925	0.925
HWE-P	0.632	0.463											

AOR was adjusted by age of participants. False discovery rate (FDR)-adjusted *P* value

RIF recurrent implantation failure, *95% CI* 95% confidence interval

Table 3 Allele combination frequencies for the *TCN2* and *TCblR* gene polymorphisms in RIF patients and control subjects

Allele combination	Controls ($2n = 394$)	Cases ($2n = 220$)	OR (95% CI)	P[a]	FDR-P
TCN2 67A>G/*TCN2* 776C>G/*TCblR* 1104C>T					
A-C-C	176 (44.7)	104 (47.3)	1.000 (reference)		
A-C-T	26 (6.7)	3 (1.4)	0.195 (0.058–0.661)	0.004	0.018
A-G-C	172 (43.6)	94 (42.7)	0.925 (0.652–1.312)	0.661	1.000
A-G-T	17 (4.2)	11 (5.0)	1.095 (0.494–2.428)	0.823	1.000
G-C-C	1 (0.4)	0 (0.0)	0.563 (0.023–13.960)	1.000	1.000
G-C-T	0 (0.0)	1 (0.5)	5.067 (0.204–125.600)	1.000	1.000
G-G-C	1 (0.4)	7 (3.2)	11.850 (1.437–97.690)	0.006	0.018
TCN2 67A>G/*TCN2* 776C>G					
A-C	203 (51.4)	108 (49.1)	1.000 (reference)		
A-G	188 (47.8)	104 (47.3)	1.040 (0.744–1.453)	0.819	1.000
G-C	1 (0.4)	0 (0.0)	0.625 (0.025–15.490)	1.000	1.000
G-G	2 (0.4)	8 (3.6)	7.519 (1.568–36.040)	0.005	0.015
TCN2 67A>G/*TCblR* 1104C>T					
A-C	348 (88.3)	198 (90.0)	1.000 (reference)		
A-T	43 (10.9)	14 (6.4)	0.572 (0.305–1.072)	0.078	0.117
G-C	3 (0.8)	7 (3.2)	4.101 (1.048–16.040)	0.043	0.117
G-T	0 (0.0)	1 (0.5)	5.267 (0.213–130.000)	0.364	0.364
TCN2 776C>G/*TCblR* 1104C>T					
C-C	178 (45.1)	104 (47.1)	1.000 (reference)		
C-T	26 (6.7)	4 (2.0)	0.263 (0.089–0.776)	0.009	0.027
G-C	173 (44.0)	101 (46.1)	0.999 (0.708–1.410)	0.997	0.997
G-T	17 (4.2)	11 (4.8)	1.107 (0.500–2.456)	0.802	0.997

Exclude criteria P value > 0.1. AOR was adjusted by age of participants. False discovery rate (FDR)-adjusted P value

RIF recurrent implantation failure, *95% CI* 95% confidence interval

[a] Chi-square test

[b] Fisher's exact test

Table 4 Genotype combination frequencies for the *TCN2* and *TCblR* gene polymorphisms in RIF patients and control subjects

Genotypes	Controls ($n = 197$)	RIF ($n = 110$)	OR (95% CI)	P	FDR-P	AOR (95% CI)	P	FDR-P
TCN2 67/*TCN2* 776								
AA/CC	51 (25.9)	27 (24.5)	1.000 (reference)			1.000 (reference)		
AA/CG	99 (50.3)	50 (45.5)	0.954 (0.536–1.699)	0.873	0.873	0.927 (0.518–1.659)	0.799	0.906
AA/GG	44 (22.3)	25 (22.7)	1.073 (0.545–2.113)	0.838	0.873	1.042 (0.525–2.067)	0.906	0.906
AG/CC	1 (0.5)	0 (0.0)	None			None		
AG/CG	1 (0.5)	4 (3.6)	7.556 (0.804–71.004)	0.077	0.154	6.274 (0.648–60.797)	0.113	0.226
AG/GG	1 (0.5)	4 (3.6)	7.556 (0.804–71.004)	0.077	0.154	7.663 (0.803–73.155)	0.077	0.226
TCN2 67/*TCblR* 1104								
AA/CC	154 (78.2)	89 (80.9)	1.000 (reference)			1.000 (reference)		
AA/CT	37 (18.8)	13 (11.8)	0.608 (0.307–1.205)	0.154	0.154	0.594 (0.299–1.180)	0.137	0.274
AA/TT	3 (1.5)	0 (0.0)	None			None		
AG/CC	3 (1.5)	7 (6.4)	4.038 (1.018–16.008)	0.047	0.094	3.845 (0.964–15.343)	0.057	0.274
AG/TT	0 (0.0)	1 (0.9)	None			None		
TCN2 776/*TCblR* 1104								
CC/CC	41 (20.8)	25 (22.7)	1.000 (reference)			1.000 (reference)		
CC/CT	10 (5.1)	2 (1.8)	0.328 (0.066–1.621)	0.171	0.513	0.297 (0.059–1.506)	0.143	0.429
CC/TT	1 (0.5)	0 (0.0)	None			None		
CG/CC	77 (39.1)	48 (43.6)	1.022 (0.553–1.890)	0.944	0.944	0.983 (0.529–1.829)	0.957	0.957
CG/CT	22 (11.2)	5 (4.5)	0.373 (0.125–1.110)	0.076	0.456	0.332 (0.109–1.008)	0.052	0.312
CG/TT	1 (0.5)	1 (0.9)	1.640 (0.098–27.408)	0.731	0.944	1.257 (0.072–21.971)	0.876	0.957
GG/CC	39 (19.8)	23 (20.9)	0.967 (0.473–1.979)	0.927	0.944	0.966 (0.467–1.999)	0.926	0.957
GG/CT	5 (2.5)	6 (5.5)	1.968 (0.543–7.127)	0.303	0.606	1.892 (0.513–6.981)	0.338	0.676
GG/TT	1 (0.5)	0 (0.0)	None			None		

RIF recurrent implantation failure, *95% CI* 95% confidence interval

Table 5 Differences of various clinical parameters according to TCN2 and TCblR gene polymorphisms in RIF women

Genotypes	Hgb (mg/dL) Mean ± SD	PLT (10^3/μL) Mean ± SD	PT (s) Mean ± SD	aPTT (s) Mean ± SD	Homocysteine (μmol/L) Mean ± SD	Folate (mg/mL) Mean ± SD	Uric acid (mg/dL) Mean ± SD	BUN (mg/dL) Mean ± SD	Creatinine (mg/dL) Mean ± SD	T.chol (mg/dL) Mean ± SD
TCN2 67A>G										
AA	12.54 ± 1.41	231.51 ± 55.44	11.04 ± 1.58	29.44 ± 3.52	6.45 ± 1.34	14.72 ± 10.75	3.96 ± 0.99	10.10 ± 2.74	0.78 ± 0.10	189.41 ± 47.51
AG	12.40 ± 1.75	249.25 ± 85.44	11.60 ± 0.58	27.86 ± 2.97	6.71 ± 0.77	23.00 ± 22.33	3.68 ± 0.85	11.89 ± 3.97	0.78 ± 0.10	191.63 ± 22.72
GG	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
P[a]	0.794	0.407	0.323	0.223	0.795	0.323	0.542	0.091	0.88	0.897
TCN2 776C>G										
CC	13.16 ± 0.90	248.35 ± 68.49	11.15 ± 0.54	29.50 ± 3.41	5.39 ± 0.38	14.29 ± 8.29	3.69 ± 0.99	10.19 ± 2.76	0.80 ± 0.10	194.89 ± 52.89
CG	12.52 ± 1.53	235.43 ± 55.60	10.93 ± 2.08	29.35 ± 3.19	6.40 ± 1.35	16.53 ± 13.04	3.97 ± 0.94	10.20 ± 2.86	0.79 ± 0.11	187.02 ± 39.18
GG	11.96 ± 1.45	213.79 ± 47.09	11.33 ± 0.70	29.07 ± 4.20	7.58 ± 0.76	12.27 ± 8.36	4.12 ± 1.07	10.36 ± 3.11	0.75 ± 0.08	188.78 ± 50.38
P[a]	0.008	0.081	0.551	0.902	0.006	0.69	0.537	0.968	0.138	0.784
TCblR 1104C>T										
CC	12.69 ± 1.32	236.51 ± 58.40	11.18 ± 1.22	29.34 ± 3.55	6.41 ± 1.30	15.56 ± 12.04	3.98 ± 1.01	10.27 ± 2.91	0.79 ± 0.10	190.67 ± 47.56
CT	11.73 ± 1.57	201.69 ± 43.22	10.25 ± 3.27	29.22 ± 3.28	6.68 ± 1.61	14.85 ± 6.20	3.57 ± 0.79	9.83 ± 2.74	0.73 ± 0.09	181.82 ± 33.77
TT	8.60 ± 0.00	306.00 ± 0.00	11.10 ± 0.00	28.20 ± 0.00	7.25 ± 0.00	7.21 ± 0.00	4.20 ± 0.00	11.60 ± 0.00	0.70 ± 0.00	182.00 ± 0.00
P[a]	0.001	0.056	0.19	0.946	0.783	0.778	0.61	0.797	0.126	0.826

RIF recurrent implantation failure, *aPTT* activated partial thromboplastin time, *PLT* platelet, *PT* prothrombin time, *T.chol* total cholesterol, *BMI* body mass index, *FSH* follicle stimulating hormone, *Hct* hematocrit, *Hgb* hemoglobin, *HTN* hypertension, *LH* luteinizing hormone

[a] One-way analysis of variance test

[b] Kruskal-Wallis test

and methylation [44]. A methionine deficiency in rat embryo cultures can lead to disturbed morphogenesis, especially the development of NTDs [45].

TCN2 and TCblR play an important role in Cbl absorption into the cell. *TCN* and *TCblR* polymorphisms are known risk factors for RPL and NTD. We designed this case–control study to investigate the association of *TCN2* and *TCblR* polymorphisms with RIF susceptibility. Homocysteine may affect the transferred IVF embryo development. Moreover, alterations in folate or Cbl metabolism, attributed to either a dietary deficiency or a genetic pre-disposition, appear to increase the risk of several pregnancy complications [46, 47]. Previous studies have reported increased plasma homocysteine levels in *TCN2* 776C>G heterozygote subjects [48].

In the present study, the G allele of *TCN2* 67 was a risk factor for RIF. Some previous studies have reported that the *TCN2* 67A>G or *TCN2* 776C>G polymorphism affects RPL risk [49, 50]. Interestingly, the *TCN2* 67 polymorphism caused alteration of amino acid such as isoleucine (Ile) to valine (Val). Moreover, this 23rd amino acid is included in the Cbl-binding motif. Although both Ile and Val have similar characteristics such as being nonpolar and hydrophobic, their alteration may affect the Cbl-binding efficiency. Unfortunately, there is no direct evidence concerning the change in the Cbl-binding efficiency by *TCN2* 67 amino acid alteration. However, the report about two plant proteins [51] have highly similar sequences and increased the possibility of an effect of *TCN2* 67 amino acid alteration. It was also reported that the *TCN2* 67A>G polymorphism affects serum holotranscobalamin (holoTC) levels, an active form of Cbl, in healthy, middle aged men and women [52]. The holoTC is Cbl, which binds TCN2 and is available for cellular uptake. Additionally, both the RIF patient group and control group have no frequency concerning the GG genotype of the *TCN2* 67 polymorphism. If the *TCN2* 67 polymorphism definitely affects the Cbl-binding efficiency, individuals with the GG genotype would have a very low ability to undergo cellular Cbl uptake. Therefore, supplementation of Cbl and folate would be less conducive to increasing plasma holoTC levels in the group with the AG type of *TCN2* 67 polymorphism. Consequently, the woman with the AG type of *TCN2* 67 polymorphism may need to consume more Cbl than the recommended dietary allowance. Although we could not observe a correlation between Cbl or holoTC and the *TCN2* 67 genotype in this study, we expect that the *TCN2* 67 polymorphism may affect the plasma Cbl and holoTC levels. Among these women, those who received IVF treatment could not provide a good environment for embryo development.

The present study demonstrated that the G allele and the AG genotype of *TCN2* 67 A>G polymorphism was associated with an increased risk for RIF patients through the case–control study. However, our study has some limitation, including the small population size and lack of the functional studies of the *TCN2* 67 A>G polymorphism. Nevertheless, several previous studies support our present study. Moreover, the present study suggests that the *TCN2* 67 A>G polymorphism may be a potential marker of plasma active Cbl (holoTC) levels and RIF susceptibility.

Conclusions

To the best of our knowledge, this is the first study to investigate the associations between *TCN2* 67A>G, *TCN2* 776C>G, and *TCblR* 1104C>T polymorphisms and the prevalence of RIF in the Korean population. We demonstrated that the *TCN2* 67A>G polymorphism was associated with an increased risk for RIF. Additionally, several allele combinations that contained the G allele of the *TCN2* 67A>G polymorphism and some of the genotype combinations containing the AG type of the *TCN2* 67A>G polymorphism were associated with RIF risk. Our study suggests a potential role for the *TCN2* 67A>G polymorphism in Korean women with RIF. However, the role of the *TCN2* and *TCblR* polymorphisms in those proteins and the pathogenesis of RIF need to be further clarified. Additionally, our findings support a potential role for *TCN2* and *TCblR* in RIF among Korean women.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of The Institutional Review Board of CHA Bundang Medical Center (Seongnam, South Korea) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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