ORIGINAL ARTICLE: EARLY PREGNANCY

Association of methionine synthase and thymidylate synthase genetic polymorphisms with idiopathic recurrent pregnancy loss

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Objective: To investigate the association between one-carbon metabolism and recurrent pregnancy loss (RPL). One-carbon metabolism is important for maintaining pregnancy, and the enzymes codified by these genes are relevant to this metabolic pathway.
Design: Case-control study.
Setting: An urban university-based hospital in South Korea.
Patient(s): A cohort of 353 RPL patients (3.09 $\pm$ 1.65 pregnancy losses) and 226 control subjects.
Intervention(s): None.
Main Outcome Measure(s): Genotyping was assessed by polymerase chain reaction–restriction fragment length polymorphism assay. We examined polymorphisms in four genes: methionine synthase (*MTR*); methionine synthase reductase (*MTRR*); methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*); and thymidylate synthase (*TS*).
Result(s): The *MTR* 2756AA polymorphism was associated with RPL. Gene-gene interaction analysis revealed that the frequency of the *MTR* 2756A–*TS* 6-bp allele combination was significantly higher in RPL.
Conclusion(s): Based on these results, we propose that the *MTR* 2756AA genotype and *MTR* 2756A–*TS* 6-bp allele combination are possible predisposing factors for RPL development in Korean women. (Fertil Steril® 2013;■:■–■. ©2013 by American Society for Reproductive Medicine.)
Key Words: One-carbon metabolism, homocysteine, polymorphism, recurrent pregnancy loss

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Recurrent pregnancy loss (RPL) is a devastating problem for married couples trying to establish a family, and this problem often presents a perplexing and frustrating challenge for clinicians. RPL is usually defined as three or more consecutive pregnancy losses before a gestational age of 20 weeks; however, this condition has recently been redefined by the American Society for Reproductive Medicine (ASRM) as two or more consecutive pregnancy losses (1, 2). In addition, no significant differences in the diagnostic factors between two and three or more consecutive pregnancy losses exist (3). RPL is estimated to occur in 2%–4% of reproductive-age couples, and the known etiologic factors of RPL include parental chromosome abnormalities, uterine abnormalities, hereditary thrombophilia, endocrinology disorders, immunologic factors, and infections as well as nutritional and environmental factors (4, 5). But ~40%–55% of RPL is caused by unknown factors (2, 6–8).

One-carbon metabolism, which involves the key molecules homocysteine and folate, is important for maintaining pregnancy (9). Folate is

Received November 4, 2012; revised January 10, 2013; accepted January 15, 2013.
J.H.K. has nothing to disclose. Y.J.J. has nothing to disclose. B.E.L. has nothing to disclose. H.K. has nothing to disclose. J.E.S. has nothing to disclose. D.H.C. has nothing to disclose. W.S.L. has nothing to disclose. N.K.K. has nothing to disclose.
The first two authors contributed equally to this work.
Supported by the Korea Healthcare Technology R&D Project (grant no. A084923), Ministry for Health, Welfare, and Family Affairs, South Korea.
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Fertility and Sterility® Vol. ■, No. ■, ■ 2013 0015-0282/$36.00
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http://dx.doi.org/10.1016/j.fertnstert.2013.01.108

essential for fetal development and acts as a substrate or cofactor for many biologic reactions, such as transfer of single-carbon units, DNA synthesis, cell growth and division, metabolism of several amino acids, transmethylation, and transsulfuration (10–12). Owing to the functions of folate in DNA synthesis and cell division, the need for this factor during pregnancy increases to support increased red blood cell mass, enlargement of the uterus, and development of the placenta and fetus (13, 14). The sulfur amino acid homocysteine is a byproduct of the methionine biosynthesis pathway (12). An increase of homocysteine reflects a shortage of folate intake or an imbalance in folate metabolism (15), and this phenomenon is frequently observed in cases of neural tube defects and fetal demise (16, 17). The balance between homocysteine and folate may be a useful predictor of pregnancy outcome.

Earlier studies described a relationship between polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*), which is closely associated with the homocysteine-folate balance, and RPL (18–20). Some groups also reported a positive association between *MTHFR* variants and RPL (18, 19); however, those variants showed negative associations with RPL occurrence in many other populations, including Korean women (18, 21). Recent evidence suggests associations of genetic variants of transcobalamin II (*TCN2*), methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*), and reduced folate carrier 1 (*RFC1*) with pregnancy loss, indicating that other one-carbon metabolism genes besides *MTHFR* may also be potential genetic predisposing factors for pregnancy loss (22–25). Therefore, we sought to examine other targets involved in one-carbon metabolism, such as methionine synthase (*MTR*) 2756A>G (rs1805087), methionine synthase reductase (*MTRR*) 66A>G (rs1801394), *MTHFD1* 1958G>A (rs2236225), and thymidylate synthase (*TS*) 1494 0bp/6bp (rs16430), because these factors have not been studied extensively in RPL etiology despite being well known polymorphisms.

PATIENTS AND METHODS

Study Subjects

Blood samples were collected from 353 patients with idiopathic RPL (age range, 22–45 years; mean ± SD age, 32.80 ± 4.29 years; body mass index [BMI], 21.40 ± 3.91 kg/m^2) and 226 control subjects (age range, 23–43 years; mean ± SD age, 33.50 ± 5.88 years; BMI 21.70 ± 3.12 kg/m^2). The patients were enrolled from March 1999 to February 2010 at the Department of Obstetrics and Gynecology of CHA Bundang Medical Center (Seongnam, South Korea). Patients with a pregnancy loss were identified by hCG testing, ultrasound, and/or physical examination at <20 weeks' gestational age. All RPL patients were diagnosed on the basis of at least two consecutive pregnancy losses before 20 weeks' gestational age, according to the ASRM definition (3). The average gestational age and number of pregnancy losses were 7.32 ± 2.04 weeks and 3.09 ± 1.65 losses, respectively. RPL patients with previous live births were excluded from this study. Patients with RPL due to anatomic, hormonal, chromosomal, infectious, autoimmune, or thrombotic causes were excluded from the study group. Anatomic causes were defined as intrauterine adhesion, septate uterus, and uterine fibroids and were evaluated with the use of hysterosalpingogram, hysteroscopy, computerized tomographic scanning, and magnetic resonance imaging. Hormonal causes were defined as hyperprolactinemia, luteal insufficiency, and thyroid disease, and were evaluated using blood measurements. Chromosomal causes were defined as translocation, trisomy and triploidy, and were evaluated using karyotyping. Infectious causes were defined as *Ureaplasma urealyticum* and *Mycoplasma hominis* infections, and were evaluated using bacteriologic culture. Autoimmune causes were defined as lupus and antiphospholipid syndrome, and were evaluated using lupus anticoagulant and anticardiolipin antibodies. Thrombotic causes were defined as thrombophilia and were evaluated by deficiencies of protein C and protein S and by anti–beta-2 glycoprotein. Chromosomal analyses were carried out with the use of standard protocols (24, 26). Metaphase chromosomes were stained with the GTG-banding method, and 20 metaphases per sample were analyzed. The women in the control group were recruited from CHA Bundang Medical Center and met the following enrollment criteria: regular menstrual cycles, a history of at least one naturally conceived pregnancy, no history of pregnancy loss, and karyotype 46,XX. The Institutional Review Board of CHA Bundang Medical Center approved the study in 1999, and each of the participants gave written informed consent.

Genotyping

DNA was extracted from leukocytes with the use of a G-DEX II Genomic DNA Extraction kit (Intron Biotechnology) according to the manufacturer's instructions. The *MTR* 2756A>G polymorphism was detected by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis using a sense primer (5′-GAA CTA GAA GAC AGA AAT TCT CTA-3′) and a reverse primer (5′-CAT GGA AGA ATA TCA AGA TAT TAG A-3′). The 189-bp PCR product was then digested with 3 U *Hae*III for 16 hours at 37°C. A digestion product of 189 bp represented the AA genotype; fragments of 189 bp, 159 bp, and 30 bp represented the AG genotype; and fragments of 159 bp and 30 bp represented the GG genotype.

The *MTRR* 66A>G polymorphism was detected by PCR-RFLP analysis. The following primers were used to amplify the *MTRR* 66A>G region: forward 5′-CAG GCA AAG GCC ATC GCA GAA GAC AT-3′ and reverse 5′-CAC TTC CCA ACC AAA ATT CTT CAA AG-3′. The 150-bp product was digested with 3 U *Afl*III for 16 hours at 37°C. A restriction fragment of 150 bp represented the AA genotype; fragments of 150 bp, 123 bp, and 27 bp represented the AG genotype; and fragments of 123 bp and 27 bp represented the GG genotype.

To detect the *MTHFD1* 1958G>A genotype, PCR-RFLP analysis was performed with a sense primer (5′-CCC ACT TTG AAG CAG GAT TG-3′) and an antisense primer (5′-CAT CCC AAT TCC CCT GAT G-3′). The length of the amplified

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