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Rs4265085 in *GPER1* gene increases the risk for unexplained recurrent spontaneous abortion in Dai and Bai ethnic groups in China

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KEY MESSAGE

A significant genetic association between rs4265085 of the G protein-coupled oestrogen receptor 1 gene and recurrent spontaneous abortion in Dai and Bai ethnic groups may reveal a new susceptibility locus in minorities in China.

A B S T R A C T

Oestrogen receptors are implicated in the pathogenesis of recurrent spontaneous abortion (RSA). Non-genomic oestrogen responses can be mediated by GPER. The prevalence of polymorphisms in *GPER1* gene in RSA was assessed in 747 Chinese women from Yunnan province (171 Bai, 258 Chinese Han, 234 Dai, 33 Achang and 51 Jingpo patients). Snapshot technology was used for genotyping the polymorphisms of the *GPER1* gene. The rs4265085G was significantly increased in the Dai and Bai groups versus controls (Dai: $P < 0.0001$, $P_{adj} < 0.0001$, OR 95% CI 2.34 [1.79 to 3.05]; Bai: $P = 0.0004$, $P_{adj} = 0.0012$, OR 95% CI 1.71 [1.27 to 2.31]); recessive model of rs4265085 in the Dai ($P = 0.003$, $P_{adj} = 0.009$, OR 95% CI 2.71 [1.38 to 5.30]); Bai ($P < 0.0001$, $P_{adj} < 0.0001$, OR 95% CI 3.37 [1.93 to 5.91]). Haplotype frequencies containing rs10269151G-rs4265085G-rs11544331C were separately significantly different in Dai and Bai ethnic groups (Dai: $P = 0.0002$, $P_{adj} = 0.001$, OR 95% CI = 2.12 [1.43 to 3.17]; Bai: $P = 0.005$, $P_{adj} = 0.025$, OR 95% CI = 1.82 [1.18 to 2.78]) compared with controls. The intron variant rs4265085 may confer risk for RSA in Dai and Bai ethnic groups.

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Introduction

Recurrent spontaneous abortion (RSA) or habitual abortion is defined as two or more consecutive spontaneous abortions before the 20th week of gestational age and affects about 1–5% of couples trying to have children (Garrido-Gimenez and Alijotas-Reig, 2015; Tunç et al., 2016). Complex causative factors, including genetic factors, immune dysfunction, maternal infections, endocrine disease, anatomical defects and environmental toxicity, are considered to be causes of RSA (Markoff et al., 2016).

Oestrogens are major endocrine hormones, playing a crucial role throughout the entire pregnancy with effects on fetal development, uteroplacental blood flow, implantation and biosynthesis of progesterone (Delbe's et al., 2006; Pepe and Albrecht, 1995; Wang et al., 2010). The oestrogen receptors alpha and beta (ERα and ERβ) are classical nuclear receptors that mediate the effects of oestrogens (Anousha et al., 2013). In addition, it is well known that oestrogens can induce immediate non-genomic or membrane effects via pathways involving G proteins and classical second messengers (Pietras and Szego, 1977). The G protein-coupled oestrogen receptor (GPER) (GPR30) was reported to regulate non-genomic oestrogen responses (Olde and Leeb-Lundberg, 2009), and is distributed throughout the human body in brain, liver, heart, lung, pancreas, placenta, blood vessels, bone, lymphoid tissue, as well as in endometrial, ovarian and breast cancers (Zhang et al., 2008). Increasing oestrogen activation of GPER in oestrogen-receptor-negative, GPER-positive breast cancer cells can be found (Filardo et al., 2002). Subsequently, GPER was identified to be a single, high-affinity, saturable and a low capacity oestrogen-binding site oestrogen receptor (Thomas et al., 2005).

Research has detected GPER in female reproductive tissues, such as breast, uterus and ovaries (Ignatov et al., 2013; Kolkova et al., 2010; Sjöström et al., 2014; Weißenborn et al., 2014). The GPER may be involved in oestrogen responses, which are fundamental in female reproduction and gynaecologic carcinogenesis. Genetic factors are a cause of RSA and include chromosomal abnormalities, gene polymorphisms and abnormal epigenetic modifications. The polymorphisms associated with RSA have mainly been sought and described in the regions of the genome encoding miRNAs and oestrogen receptors (Alessio et al., 2008; Hu et al., 2011; Jeon et al., 2012; Kim et al., 2015; Li et al., 2016; Wang et al., 2004). No research, however, has been conducted on the association between *GPER1* gene polymorphisms and RSA. Therefore, the aim of this study was to investigate the association of *GPER1* polymorphisms in five ethnic groups who had experienced RSA in China.

Materials and methods

Participants

The study protocol was approved by the Ethics Committee of Changsha Medical University (EC/15/023, 03/09/2015). Informed written consent was obtained from all participants. Written informed consent for genetic analysis were obtained from all participants or their guardians. The eligibility criteria for recruitment into the RSA group included women without systematic disease, abnormal menstrual period, abnormal karyotype, abnormal semen (partners), positive results for auto-anti-sperm, anti-nucleus, or anti-phospholipid antibodies, anatomical defects of reproductive organs, infection of the reproductive tract, and who had experienced at least two consecutive first-trimester pregnancy losses and had no living children. A total of 747 women (28.5 ± 0.6 years) who had experienced RSA were recruited, made up of 171 Bai (29.3 ± 0.3 years), 258 Chinese Han (25.1 ± 0.2 years), 234 Dai (26.2 ± 0.6 years), 33 Achang (28.8 ± 0.4 years) and 51 Jingpo (27.2 ± 0.7 years) patients. Of the 747 patients in the RSA group, 160 Bai, 233 Chinese Han, 221 Dai, 30 Achang and 46 Jingpo ethnic patients had experienced two first-trimester pregnancy losses and 11 Bai, 25 Chinese Han, 13 Dai, 3 Achang and 5 Jingpo had experienced more than two first-trimester pregnancy losses. The samples were collected from three different hospitals (Kunhua Hospital, Dehong People's Hospital and the First Affiliated Hospital of Changsha Medical University). In addition, 779 healthy controls (31.6 ± 0.4 years) including 179 Bai (28.6 ± 0.3 years), 264 Chinese Han (26.5 ± 0.7 years), 240 Dai (24.5 ± 0.8 years), 39 Achang (27.8 ± 0.6 years) and 57 Jingpo (28.3 ± 0.6 years) individuals matched by ethnicity and age who were not in their first pregnancy, had at least one living child, had no history of spontaneous abortion or stillbirth, no systematic disease, no infection of the reproductive tract, and no genetic, endocrinological or anatomical abnormalities, were also recruited.

Genotyping

Three polymorphisms (rs10269151, rs4265085 and rs11544331) of *GPER1* gene were selected. Selection was based on known functional effects or previous reported associations with disease phenotypes (Chevalier et al., 2014; Giess et al., 2010; Peng et al., 2012). The standard phenol–chloroform method was used in extraction of genomic DNA. Multiplex polymerase chain reaction (PCR) was carried out on a GeneAmp 9700 PCR thermocycler (Applied Biosystems, Foster City, California, USA). Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (EXO I) were used to remove primers and unexhausted dNTPs from the PCR product, which was purified with 2 μl of SAP/EXO mix (contain 2 IU SAP and 2 IU Exo I) for 1 h at 37°C, followed by 15 min at 75°C to inactivate the enzyme. A 5 μl reaction volume (containing 0.5 μl SNaPshot MIX, 1 μl SNaPshot primer and 3 μl purified PCR products) was used in single base extension on a GeneAmp 9700 PCR thermocycler (Applied Biosystems, Foster City, California, USA). Cycling parameters were as follows: 95°C denaturation for 15 s, annealing at 55° C for 5 s and extension at 60°C for 30 s, 15 min at 75°C for enzyme inactivation and maintained at 4°C until analysis. A total of 0.5 IU of serum alkaline phosphatase was used in 5 μl single base extension product for 1 h at 37°C, followed by 15 min at 75°C for enzyme inactivation and maintained at 4°C until analysis. Sample analysis was conducted in a 10 μl reaction (containing 1 μl mini sequencing products, 6.5 μl Hi-Di formamide and 0.5 μl of GeneScan LIZ-120ABI) on the 3730XL genetic analyzer (Applied Biosystems, Foster City, California, USA). The resulting data were analysed by GeneScan ™3.7 Software (Applied Biosystems, Foster City, California, USA). The primers used for genotyping are detailed in **Table S1**.

Statistical analysis

Allele and genotype frequencies of *GPER1* polymorphisms were compared between patients and controls using a two-sided Fisher's exact test. The Hardy–Weinberg equilibrium calculations, allele and genotype frequencies, haplotype analyses, and *P*-value permutation were conducted by using PLINK 1.09 (http://pngu.mgh.harvard.edu/~purcell/plink/) (Purcell et al., 2007). The corresponding odds ratios