**ORIGINAL ARTICLE**

**Infertility**

**BDNF Val66Met polymorphism is associated with Stage III–IV endometriosis and poor in vitro fertilization outcome**

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**BACKGROUND:** The recently identified human brain-derived neurotrophic factor (BDNF) Val66Met polymorphism was found to be associated with altered susceptibility to some neuropsychiatric disorders. Interestingly, BDNF together with its receptors TrkB and p75 are extensively expressed in female reproduction system. The aim of this study is to investigate whether the BDNF Val66Met polymorphism plays a role in endometriosis, endometriosis-related infertility and the outcomes of IVF and embryo transfer (IVF–ET).

**METHODS:** A case–control study included 425 endometriosis patients and 244 control Chinese Han women. The genotyping of the BDNF Val66Met polymorphism was performed by the fluorescence resonance energy transfer method. The plasma and follicular fluid concentrations of BDNF on the day of oocyte retrieval were measured by ELISA. The general clinical data from the endometriosis-related and tubal obstructed infertile patients treated with IVF–ET were analyzed.

**RESULTS:** There was no association between the BDNF Val66Met polymorphism and overall endometriosis (P > 0.05), whereas higher genotype and allele frequencies of the BDNFMet polymorphism were found in the Stage III–IV endometriosis (both P < 0.01) and endometriosis-related infertile patients (both P < 0.05). Moreover, during IVF and embryo transfer (IVF–ET) treatment, fewer mature oocytes (P < 0.05) and lower fertilization rate (P < 0.01) were found in BDNFMet/Met carriers compared with those in BDNFVal/Val carriers with infertility. Follicular-fluid BDNF concentration in BDNFMet/Met carriers was lower compared with that in BDNFVal/Val individuals (P < 0.01).

**CONCLUSIONS:** Our results suggest that the BDNFMet single-nucleotide polymorphism might contribute to the increased susceptibility to the Stage III–IV endometriosis and endometriosis-related infertility. Moreover, infertile patients with the BDNFMet/Met genotype had a poorer IVF outcome compared with the BDNFVal/Val genotype individuals, which might in part be due to the decreased BDNF levels in follicular fluids after controlled ovarian hyperstimulation.

**Key words:** endometriosis / female infertility / gene mutations / follicular fluid / IVF/ICSI outcome

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**Introduction**

Endometriosis is one of the most common benign diseases encountered in gynecology, which is defined as the presence of endometrial tissue outside the uterine cavity. Clinically, propensity to cause infertility is a distressing characteristic associated with endometriosis, and until now the mechanism underlying endometriosis and its reproductive failure remain uncertain. Familial aggregation and clinical case–control studies have revealed many genetic variants related to the immune, neuroendocrine and reproductive function play important roles in the development of endometriosis, suggesting a genetic component (Falconer et al., 2007).

Brain-derived neurotrophic factor (BDNF) has well been recognized for its trophic effects on neuronal survival, neurogenesis and synaptic
plasticity (Cowansage et al., 2010). In humans, the BDNF gene is located on the short arm of chromosome 11 (11p13). Recently, a BDNF gene 196G/A polymorphism (dbSNP: rs6265) was identified, which results in an amino acid substitution (Val/Met) in position 66 at BDNF prodomain and leads to altered intracellular trafficking and decreased regulated secretion of BDNF in neurons (Egan et al., 2003; Chen et al., 2004). A series of genetic association studies have linked the BDNF Val66Met polymorphism with altered susceptibility to neuropsychiatric disorders, such as depression, schizophrenia, bipolar disorder and Alzheimer’s disease (Chen et al., 2008; Frielingdorf et al., 2010).

BDNF together with its high-affinity tyrosine kinase type B (TrkB) receptor and pan-neurotrophin low-affinity receptor p75 (p75NTR) were found to be extensively expressed in mammalian female reproductive system (Seifer et al., 2002; Krizsan-Agbas et al., 2003; Paredes et al., 2004). Moreover, plasma and follicular-fluid BDNF levels exhibit dynamic changes during the menstrual cycle in normally cycling fertile women or in the process of controlled ovarian stimulation for IVF (Seifer et al., 2003; Begliuomini et al., 2007; Monteleone et al., 2007). These findings suggested that BDNF may also play a role in female reproduction. Loss-of-function and gain-of-function experiments revealed significant roles of BDNF in steroidogenesis, folliculogenesis, early follicle development, polar bodies extrusion and ovulation (Jensen and Johnson, 2001; Seifer et al., 2002; Kawamura et al., 2005; Lee et al., 2007; Dissen et al., 2009). Furthermore, BDNF is known to up-regulate antioxidants and block the development of oxidative stress (Altar et al., 1994; Mattson et al., 2002; Boutahar et al., 2010). It has been suggested that reactive oxygen species or free radicals, the major factors leading oxidative stress, may promote the growth and adhesion of endometrial cells in the peritoneal cavity and lead to endometriosis and infertility (Szczepanska et al., 2003; Jackson et al., 2005; Augoulea et al., 2009). Thus, not only the localization of BDNF in the reproductive endocrine system, but also its potential biological activities in oxidative stress suggest a possible role of BDNF in the pathogenesis of endometriosis and its related infertility.

In the present study, we investigate whether the BDNF Val66Met polymorphism is associated with the stage of endometriosis and endometriosis-related infertility. Further, we assess whether infertile patients carrying the BDNF<sup>Val<sub>66Met</sub></sup> genotype have a poorer IVF–ET treatment outcome compared with BDNF<sup>Val<sub>66Val</sub></sup> individuals.

### Materials and Methods

#### Patients and controls

This study was performed in accordance with the guidelines in the Declaration of Helsinki and has been formally approved by the Medical Ethics Committee of Shandong University and the Second Hospital Affiliated to Shandong University of Traditional Chinese Medicine. All 669 unrelated Chinese Han women, who were aged 22–40 years and undergoing laparoscopy either to investigate subfertility or for laparoscopic sterilization were recruited in the Second Hospital Affiliated to Shandong University of Traditional Chinese Medicine and Qilu Hospital of Shandong University from 2010 to 2011. The control group consisted of 244 non-endometriosis women (mean age: 32.1 ± 3.7 years), which included 114 healthy fertile women and 130 infertile women caused by tubal obstruction. Infertility was defined as unsuccessful attempts to become pregnant after at least 24 consecutive months of unprotected intercourse. Case patients consisted of 425 women (mean age: 31.2 ± 3.1 years) with histologically and laparoscopic confirmed endometriosis. The disease stages according to the revised American Society for Reproductive Medicine classification of endometriosis (ASRM, 1997) were as follows: Stages I–II (minimal to mild), 169 patients and Stages III–IV (moderate to severe), 256 patients. In the case group, 145 patients were fertile and 280 patients were infertile with endometriosis as the only reason for infertility. In the group of fertile women with endometriosis, there were 68 patients with Stage I–II endometriosis and 77 with Stage III–IV endometriosis. In the group of infertile women with endometriosis, there were 101 patients with Stage I–II (minimal/mild) endometriosis and 179 with Stage III–IV (moderate/severe) endometriosis. Of all 410 infertile patients, 102 tubal obstructed infertile and 198 endometriosis-related infertile women received a standard long protocol IVF treatment and complete fresh embryos transfer in this cycle. All recruited women with polycystic ovary syndrome, diminished ovarian reserve, unexplained infertility and whose partner had any male infertile factors were excluded from the study. Informed consent was obtained from all subjects.

#### Genotype analysis

Genomic DNA was isolated from whole blood according to standard procedures. All genotypes were performed by the fluorescence resonance energy transfer (FRET) method using the Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). Forward primer: 5′-AAC ATC CGA GGA CAA GGT GG-3′; reverse primer: 5′-GGA CAT GTT TGC AGC ATC TAG GTA A-3′; donor hybridization probe: 5′-GCT CTT CTA CGT GTT CGA AAG TG-FL-3′; acceptor hybridization probe: 5′-LC640-CAG CCA ATG ATG TCA CGT CTA CGT CGT CTA AAG TG-3′ (TIB MOLBIOL, Germany). After amplification, a melting curve was generated by holding the reaction at 50°C for 30 s and then heating slowly to 70°C with a ramp rate of 0.1°C/s. Peaks were obtained at 58°C for the Met-allele and at 65°C for the Val-allele.

#### Measurement of plasma and follicular-fluid BDNF levels

Levels of BDNF were determined by using the commercially available BDNF Emax Immunoassay System (BDNF Emax<sup>®</sup> ImmunoAssay System, Promega, USA). The ELISA was performed according to the manufacturer’s protocol. Briefly, 96-well plates were coated with anti-BDNF monoclonal antibody overnight at 4°C. Then, the wells were blocked with blocking buffer and incubated with follicular fluid or plasma samples and standards for 2 h at room temperature. Anti-human BDNF polyclonal antibody was used as reporter antibody, and anti-IgY–horseradish peroxidase conjugate was used to detect the amount of specifically bound polyclonal antibody. After incubation with the chromogenic substrate and stopping of the reaction with 1 N hydrochloric acid, the absorbency was measured at 450 nm by using a microplate reader (Model 680 microplate reader, Bio-Rad Laboratories Ltd, CA, USA). All samples were assayed in duplicate.

Blood samples were obtained between 7:30 a.m. and 8:00 a.m. on the day of oocyte retrieval. The blood samples were drawn into tubes containing EDTA (pH 7.5) and immediately centrifuged at 2500 g for 10 min at 4°C, and then the plasma samples were collected and stored at −80°C until assay. Follicular fluid was collected during routine egg retrieval from 198 endometriosis-related infertile patients and 102 tubal obstructed infertile women undergoing controlled ovarian stimulation in preparation for IVF. Aspirates were obtained from the first follicle from either side in an effort to obtain clear follicular fluid. After removal of the oocyte, the fluid was processed by centrifuge at 300g for 10 min, and the clear supernatant was stored at −80°C until assay.
To investigate whether the common BDNF polymorphism (Val66Met) which leads to decreased BDNF secretion plays a role in the pathogenesis of endometriosis, we initially investigated the frequency of the BDNF Val66Met polymorphism in endometriosis patients and controls in a Chinese Han population. Results are shown in Table I.

### Statistical analysis

Statistical analyses were performed using SPSS for Windows 13.0 (SPSS, Inc., Chicago, IL, USA). The χ² analysis was used to compare allele and genotype frequencies among the groups and to estimate the Hardy–Weinberg equilibrium. For reducing the chance of type 1 error, the Bonferroni correction was used to adjust P-values when multiple comparisons were conducted in the IVF cycles undergoing fresh embryos transfer. The level of significance was set at 0.05.

### Results

#### Table I Frequency distribution of BDNF Val66Met genotypes and alleles in endometriosis patients and controls.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>BDNF Val66Met genotypes, n (%)</th>
<th>P-value*</th>
<th>OR (95% CI) a</th>
<th>Allele frequencies, n (%)</th>
<th>P-value*</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V/V</td>
<td>M/V</td>
<td>M/M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n = 244)</td>
<td>78 (32.0)</td>
<td>107 (43.9)</td>
<td>59 (24.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometriosis (n = 425)</td>
<td>108 (25.4)</td>
<td>198 (46.6)</td>
<td>119 (28.0)</td>
<td>0.174</td>
<td>1.38</td>
<td>0.98–1.95</td>
</tr>
<tr>
<td>Stage I–II (n = 169)</td>
<td>61 (36.1)</td>
<td>73 (43.2)</td>
<td>35 (20.7)</td>
<td>0.0001 (0.002)</td>
<td>2.09</td>
<td>1.38–3.17</td>
</tr>
<tr>
<td>Stage III–IV (n = 256)</td>
<td>47 (18.4)</td>
<td>125 (48.8)</td>
<td>84 (32.8)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

P-values in the parenthesis were corrected by the Bonferroni correction.

OR, odds ratio; CI, confidence interval

a Versus control.
The power analysis showed that the smallest sample size still had a power of 0.962 to detect significant genotypic associations and a power of 0.999 to detect allelic associations, given that an effect size index of 0.2 (corresponding to ‘weak to moderate’ gene effect) was used. The BDNF Val66Met genotype distributions in four groups agreed with the Hardy–Weinberg equilibrium ($\chi^2 = 3.374$ for control, $P = 0.066$; $\chi^2 = 1.943$ for endometriosis, $P = 0.16$; $\chi^2 = 2.241$ for Stage I–II endometriosis, $P = 0.13$; $\chi^2 = 0.002$ for Stage III–IV endometriosis, $P = 0.97$, all $P > 0.05$). Compared with the control group, the frequencies of BDNF Val66Met genotypes and Met allele had trends to increase in endometriosis patients, but did not reach statistical significance ($\chi^2 = 3.5, P = 0.174$ and $\chi^2 = 3.338, P = 0.068$, respectively). Interestingly, when endometriosis patients were divided according to their disease severity, the frequency of BDNF Met polymorphism was significantly higher after the Bonferroni correction in Stages III–IV (genotype: $\chi^2 = 13.175$, $P_{\text{correction}} = 0.002$, OR = 2.09, 95% CI = 1.38–3.17; allele: $\chi^2 = 12.374$, $P_{\text{correction}} < 0.001$, OR = 1.56, 95% CI = 1.22–2.01) but not in Stages I–II (genotype: $\chi^2 = 1.044$, $P_{\text{correction}} = 1.00$; allele: $\chi^2 = 1.167$, $P_{\text{correction}} = 0.56$) endometriosis patients compared with the control group (Table I), suggesting Met allele confers an increased risk for Stage III–IV endometriosis.

**Distribution of BDNF Val66Met polymorphism in fertile and infertile patients**

Propensity to cause infertility is a distressing characteristic associated with endometriosis. When endometriosis patients were classified based on fertile status, the infertility percentage was significantly higher in Stage III–IV endometriosis patients (69.9%) compared with that in Stage I–II endometriosis patients (59.8%) ($\chi^2 = 4.674, P = 0.031$). Since we have found that BDNF Met polymorphism could contribute to the increased risk for Stage III–IV endometriosis, we speculated that this single-nucleotide polymorphism (SNP) might also be associated with the endometriosis-related infertility. In control population, the frequencies of BDNF Val66Met genotypes and allele showed no statistically difference between the fertile and infertile subgroups ($\chi^2 = 1.69, P = 0.43$ and $\chi^2 = 0.869, P = 0.351$, respectively). However, compared with those in control infertile women, higher BDNF Met/Met genotype ($\chi^2 = 6.285, P = 0.043$, OR = 1.79, 95% CI = 1.10–2.91) and Met allele frequencies ($\chi^2 = 5.880, P = 0.015$, OR = 1.44, 95% CI = 1.07–1.94) were shown in endometriosis-related infertile patients (Table II). These results suggested that the BDNF Val66Met polymorphism was associated with endometriosis-related infertility.

**BDNF Val66Met polymorphism and the IVF outcome in endometriosis-related and tubal obstructed infertile patients**

We further analyzed the effect of the BDNF Val66Met polymorphism on IVF outcomes of 198 endometriosis-related infertile patients, whose control were 102 tubal obstructed infertile patients. No significant differences were found in some indexes between endometriosis-related infertile patients with different BDNF Val66Met genotypes, such as age, BMI, basal FSH levels, number of oocyte retrieved per cycle, implantation rate, pregnancy rate and endometrial thickness at HCG day (all $P > 0.05$). However, though administered with higher doses of gonadotrophins ($P < 0.01$), patients with the BDNF Val/Val genotype had achieved fewer mature oocytes ($P < 0.01$), lower fertilization rate ($P < 0.01$) and lower good quality rate of embryos ($P < 0.05$) than those in BDNF Val/Met and BDNF Met/Met carriers (Table III). Similarly, the differences in the dose of gonadotrophins usage ($P < 0.01$), the number of mature oocytes ($P < 0.05$) and fertilization rate ($P < 0.01$) were also obtained in tubal obstructed infertile patients between BDNF Val/Val and BDNF Met/Met carriers.
women with IVF treatment. No significant differences were found in plasma BDNF levels among different BDNF genotypes in tubal obstructed and endometriosis-related infertile groups [Fig. 1A, for tubal obstructed infertile women, F(2, 67) = 0.762, P = 0.471; for endometriosis-related infertile women, F(2, 197) = 1.939, P = 0.147]. However, follicular-fluid BDNF levels in different BDNF Val66Met genotypes showed a significant difference in both groups [Fig. 1B, for tubal obstructed infertile women, F(2,101) = 14.82, P < 0.001; for endometriosis-related infertile women, F(2,197) = 6.539, P = 0.002]. BDNF Met/Met carriers having significantly decreased follicular-fluid BDNF levels compared with that in BDNF Val/Val patients (both P < 0.01).

### Discussion

The purpose of this study is to investigate whether the BDNF Val66Met polymorphism is associated with the altered susceptibility to endometriosis and its related infertility. We provide evidence that the BDNF Met allele confers an increased risk for Stage III–IV endometriosis and endometriosis-related infertility. Moreover, we found that both endometriosis-related and tubal obstructed infertile patients with the BDNF Met/Met genotype had a poor IVF outcome, which might in part be due to the decreased BDNF levels in follicular fluids.

The frequency of the BDNF Met allele is relatively common in Asian populations, ~40–50% of which carry at least one BDNF Met allele (Shimizu et al., 2004; Pivac et al., 2009). Because of the wide expression and important role of BDNF in the central nervous system, the studies on the association between the BDNF Val66Met polymorphism and neuropsychiatric disorders have been extensively reported. Moreover, a few studies about the role of BDNF Val66Met in non-neuronal system diseases, such as unstable angina (Jiang et al., 2009), allergic asthma and allergic rhinitis (Andiappan et al., 2011), have also been recently published. Our data provide several novel insights into the association of the BDNF Val66Met polymorphism with a gynecological and obstetric disorder, endometriosis, in Chinese Han population. First, we found a significant increased frequency of the BDNF Met allele in the Stage III–IV endometriosis patients. As most Stage III–IV endometriosis could lead to infertility, we next found higher BDNF Met/Met genotype and Met allele frequencies in endometriosis-related infertile patients compared with those in tubal obstructed infertile patients. However, it is still unclear that the association between the BDNF Val66Met variant and endometriosis-related infertility is a coincidental finding along with Stage III–IV endometriosis or whether this polymorphism has an independent role in the reproductive endocrine system, except endometriosis. In the present study, we identified the BDNF Val66Met polymorphism as a newly confirmed genetic risk factor for endometriosis and endometriosis-related infertility.

Secondly, we found decreased follicular-fluid BDNF levels in BDNF Met/Met carriers compared with that in BDNF Val/Val carriers in both the endometriosis-related and tubal obstructed infertile patients during IVF treatment. To the best of our knowledge, our data show for the first time that BDNF Met/Met variant could influence BDNF levels in human ovarian follicles. A previous study by Buyuk and Seifer (2008) found that women with a history of endometriosis had significantly lower follicular-fluid BDNF levels compared with those with male-factor (control) infertility. Considering that the frequency of the BDNF Met variant is high in infertile women with endometriosis, we think that the low follicular BDNF levels induced by the BDNF Met/Met variant may be a reasonable explanation for endometriosis-related infertility. It has been shown that human cumulus granulosa cells produce and secrete BDNF into follicular fluid (Seifer et al., 2002). Moreover, BDNF levels in follicular fluids or granulosa cell culture media appeared to be up-regulated by gonadotrophin stimulation...
administration of BDNF could improve the development of early follicles, extrusion of first polar body, cytoplasmic maturation of oocytes and growth of preimplant embryo (Seifer et al., 2002; Paredes et al., 2004; Kawamura et al., 2005; Lee et al., 2007). Thus, the blunted ovarian response to gonadotrophins might be due to the decreased follicular-fluid BDNF levels in the BDNF<sub>Met/Met</sub> variant. In addition, BDNF<sub>Met/Met</sub> carriers had decreased mature oocyte number, fertilized rate and good quality rate of embryos; however, there was no difference in the pregnancy rate in the current cycle of fresh embryos implantation as the good embryos were selected for transplantation. As we know, the pregnancy rate per IVF–ET cycle is only around 40–50%, which means that more than half of the patients need multiple rounds of embryo transplantations. For the BDNF<sub>Met/Met</sub> patients, the chance for the subsequent embryo transplantation is reduced because fewer good quality embryos could be obtained, which suggests that the BDNF<sub>Met/Met</sub> patients have increased risk to undergo the expensive, invasive and stressful IVF–ET treatment from the beginning again. Moreover, we found that decreased follicular-fluid BDNF levels were associated with the poor outcomes of IVF, which suggested that supplying exogenous BDNF to oocytes’ culture medium during IVF may be a helpful method in the future to improve the IVF outcome in infertile patients carrying the BDNF<sub>Met/Met</sub> variant.

Finally, the present study also has some limitations. First, as a newly confirmed genetic risk factor, the BDNF<sub>Val66Met</sub> polymorphism showed positive correlations with endometriosis, endometriosis-related infertility and poor outcomes of IVF–ET. However, the mechanisms underlying this association are still unclear. A BDNF<sub>Met</sub> knock-in transgenic mouse was recently generated and provided a unique tool to study the in vivo consequences of BDNF<sub>Met</sub> SNP (Chen et al., 2006). In the future, it will be interesting to investigate the role of BDNF<sub>Met</sub> SNP in increasing growth and adhesion of endometrial cells in the peritoneal cavity, folliculogenesis and oocyte maturation using the BDNF<sub>Met</sub> knock-in mouse. Secondly, since the data were collected from two hospitals in Jinan area, the results may not be generalizable to the entire population. Moreover, though all the women recruited in this study including the endometriosis group and the control group have undergone laparoscopic examination, we still cannot exclude the possibility that some asymptomatic women with endometriosis might be enrolled in the control group.

In the future, it is necessary to replicate and establish this association in multicenter with more samples and strict diagnostic criteria for endometriosis before its prognostic value can be suggested with certain.

In conclusion, this study presents the first evidence that the BDNF<sub>Val66Met</sub> polymorphism is associated with the Stage III–IV endometriosis and endometriosis-related infertility in Chinese Han population. Moreover, this SNP can lead to poor IVF outcomes both in endometriosis-related and in tubal obstructed infertile women, which further confirms that BDNF play an important role in female reproduction system.

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Authors’ roles

Z.Y.C. supervised the whole study procedure, including conception, design and critical revisions of the draft manuscript. Q.Y.Z. and Q.G. provided IVF patient data, did statistical analyses and contributed to interpretation of the data. Y.W. and X.F. designed, performed and analyzed the genotyping and ELISA experiments, wrote the manuscript. W.S., J.W. and W.C. were responsible for the patients recruit and diagnosis. F.Y.K. and Y.Y. contributed to the samples collection of blood and follicular fluid. All authors participated in the ultimate interpretation of the study data and approved the final manuscript.

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Conflict of interest

There is no conflict of interest in this manuscript.

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