Association between genetic polymorphisms in fibroblast growth factor (FGF) 1 and FGF2 and risk of endometriosis and adenomyosis in Chinese women

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BACKGROUND: Angiogenesis appears to be an important event in the pathophysiology of endometriosis (EM) and adenomyosis. Two angiogenic factors, fibroblast growth factor (FGF) 1 and 2, play a central role in the initiation of angiogenesis. We investigated whether FGF1 -1385A/G and FGF2 754C/G polymorphisms are associated with a risk of developing EM and adenomyosis.

METHODS: Genotypes were analyzed by the PCR–restriction fragment length polymorphism method in two groups of women, of Han ethnicity in north China, aged 16–55 years: (1) 421 EM patients and 421 controls; (2) 269 adenomyosis patients and 269 controls.

RESULTS: There was no difference in genotype distribution of the FGF1 -1385A/G polymorphism between adenomyosis cases and controls (P > 0.05), but the frequency of the A allele in EM patients was lower than that in controls (P = 0.013). Genotype and allele frequencies of the FGF2 754C/G polymorphism were significantly different in both EM and adenomyosis cases versus control groups. Compared with C/C homozygotes, the G allele (C/G + G/G) was associated with a decreased susceptibility to developing EM [odds ratio (OR) = 0.575, 95% confidence interval (CI) = 0.387–0.854] and adenomyosis (OR = 0.577, 95% CI = 0.367–0.906). Combined genotype analysis of both polymorphisms also showed differences between cases versus controls (all P < 0.001).

CONCLUSIONS: Our study shows for the first time that the FGF2 754C/G polymorphism may be associated with a risk of developing EM and adenomyosis in north Chinese women. Carriers of the G allele in the FGF2 gene appear to be protected from these gynecological diseases. Further studies in other populations, and of other candidate genes, are now warranted.

Key words: endometriosis / adenomyosis / fibroblast growth factor 1 / fibroblast growth factor 2 / single nucleotide polymorphism

Introduction

Endometriosis (EM) and adenomyosis are two common gynecological disorders characterized by the presence of endometrial glands and stroma outwith their normal locations. When endometrial glands and stroma are present outside the uterine cavity, the disease is called EM, and when present within the myometrium, the disease is defined as adenomyosis. Despite the increasing evidence supporting a genetic component to these diseases, the molecular mechanisms involved in development of EM and adenomyosis remain unknown.

Clinical observations and experimental studies have led to the view that EM and adenomyosis are invasive diseases. Analogous to tumor metastasis, endometriotic cell implants require neovascularization to establish, grow and invade (Fujishita et al., 1999; Taylor et al., 2002; Gescher et al., 2003). Factors involved in angiogenesis may thus play an important role in the development and progression of EM and adenomyosis.

Fibroblast growth factors (FGFs) are a family of growth factors involved in angiogenesis, wound healing and embryonic development. The FGF family consists of 22 members. FGF1 (or acidic FGF) and
FGF1 and FGF2 are prototypical members of the FGF family (Szébenyi and Fallon, 1999). One important function of FGF1 and FGF2 is the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures: thus they promote angiogenesis, the growth of new blood vessels from the pre-existing vasculature. FGF1 and FGF2 are more potent angiogenic factors than vascular endothelial growth factor (VEGF) or platelet-derived growth factor (Cao et al., 2003). FGF2, in particular, was actually the first purified growth factor shown to have angiogenic activity (Shing et al., 1984). Experimental approaches and clinical data have shown that both FGF1 and FGF2 may behave as transforming/oncogenic factors and may be involved in tumor progression (Forough et al., 1993; Nesbit et al., 1999; Soslow et al., 1999). In patients with EM, there is increased expression of the FGF1 and FGF2 protein in the ectopic, compared with the eutopic, endometrium (Mihalich et al., 2003; Hayrabedyan et al., 2005; Bourlev et al., 2006). Propst et al. (2001) reported that the expression of FGF2 in glandular epithelium and stroma of adenomyosis was significantly increased compared with autologous endometrium. Therefore, FGF1 and FGF2 may play a role in the development and progression of EM and adenomyosis.

Two single nucleotide polymorphisms (SNPs), -1385A/G (C/T, rs34011) and 754C/G (rs2922979), were identified in the promoter region of the FGF1 gene and in intron 1 of the FGF2 gene, respectively. These two SNPs may alter the protein expression level of FGF1 and FGF2, and are associated with developing Alzheimer's disease and oncogenic factors and may be involved in tumor progression. FGF1 and FGF2 are prototypical members of the FGF family (Szébenyi and Fallon, 1999). One important function of FGF1 and FGF2 is the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures: thus they promote angiogenesis, the growth of new blood vessels from the pre-existing vasculature. FGF1 and FGF2 are more potent angiogenic factors than vascular endothelial growth factor (VEGF) or platelet-derived growth factor (Cao et al., 2003). FGF2, in particular, was actually the first purified growth factor shown to have angiogenic activity (Shing et al., 1984). Experimental approaches and clinical data have shown that both FGF1 and FGF2 may behave as transforming/oncogenic factors and may be involved in tumor progression (Forough et al., 1993; Nesbit et al., 1999; Soslow et al., 1999). In patients with EM, there is increased expression of the FGF1 and FGF2 protein in the ectopic, compared with the eutopic, endometrium (Mihalich et al., 2003; Hayrabedyan et al., 2005; Bourlev et al., 2006). Propst et al. (2001) reported that the expression of FGF2 in glandular epithelium and stroma of adenomyosis was significantly increased compared with autologous endometrium. Therefore, FGF1 and FGF2 may play a role in the development and progression of EM and adenomyosis.

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Materials and Methods

Study subjects

Blood samples were obtained from the following four groups, and DNA was extracted for genotyping: (i) 421 inpatients with EM in the Fourth Affiliated Hospital, Hebei Medical University, between 2001 and 2009, (ii) 421 healthy female blood donors who were age-matched with EM patients, (iii) 269 inpatients with adenomyosis in same hospital between 2001 and 2009, (iv) 269 healthy female blood donors, age-matched with adenomyosis patients. The patients with EM and adenomyosis all underwent laparoscopy or laparotomy and the disease diagnosis was confirmed histologically. The EM patients were chosen from the cases with stage (iii)–(iv) disease. The stage of EM was assessed according to the revised American Fertility Society (1985) classification.

The control groups consisted of women of reproductive age with no malignant disease, and EM or adenomyosis was confirmed either by surgical exploration (Cesarean section) or pathologically after hysterectomy performed for dysfunctional uterine bleeding, or in the case of physical examination, confirmed by ultrasound examination. All of the subjects were women of Han ethnicity in north China. The Ethics Committee of Hebei Obstetrics and Gynecology Institute approved the study and informed consent was obtained from all recruited subjects.

DNA extraction

Venous blood (5 ml) was drawn from each subject to Vacutainer tubes containing EDTA and stored at 4°C. Genomic DNA was extracted within 1 week by proteinase K (Merck, Darmstadt, Germany) digestion followed by a salting out method as previously described (Miller et al., 1988).

FGF1 -1385a/g and FGF2 754c/g genotyping

Genotypes were determined by the PCR–restriction fragment length polymorphism (PCR–RFLP) method. Briefly, the PCR was performed in 20 μl volume containing 100 ng of DNA template, 2.0 μl of 10 x PCR buffer for FGF1 -1385A/G and 2.4 μl of 10 x PCR buffer for FGF2 754C/G, 1 U of Taq DNA polymerase (Tiangen Biotech Co., Ltd, Beijing, China). 0.4 μl of 10 mmol/l dNTPs and 200 nM of each primer. The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at 60°C and 45 s at 72°C, with a final step at 72°C for 7 min to allow for the complete extension of all PCR fragments. Accordingly, a 355 bp PCR amplification fragment was generated using the primers 5′-TCAAGCAATTCTCCTGCCTT-3′ (forward) and 5′-CCAC TTCAAGGATTATGGTG-3′ (reverse) for FGF1 -1385A/G, and a 334bp PCR amplification fragment using the primers 5′-AAGCGGCTGTAATGCAAAAC-3′ (forward) and 5′-GGTACTGGTTTACAGGCCAAT-3′ (reverse) for FGF2 754C/G. A 5.5 μl aliquot of every PCR product was subjected to digestion at 37°C overnight in a 10 μl reaction volume containing 5U restriction enzyme HhaI (Sangon Biotechnology Co., Ltd) for both SNPs. After the overnight digestion, the products for -1385A/G in FGF1 were separated by electrophoresis on a 3% agarose gel containing ethidium bromide, and the products for 754C/G in FGF2 were similarly separated on a 4% agarose gel. For FGF1 -1385A/G, the homozygous A/A was represented by DNA bands of 53 and 302 bp, the homozygous G/G was identified by DNA bands of 53, 141 and 161 bp, whereas the heterozygous AG displayed a combination of all the above bands (53, 141, 161 and 302 bp) (Fig. 1). The FGF2 754C/G genotype yielded fragments of 21, 38, 113 and 162 bp, whereas the G/G genotype is not cleaved at the mutated site and revealed fragments of 38, 113 and 183 bp, whereas the heterozygous CG manifested a combination of all the above bands (21, 38, 113, 162 and 183 bp) (Fig. 2).

For a negative control, distilled water was used instead of DNA in the reaction system for each panel of PCR. The PCR of 15% of the samples were run in duplicate for quality control, with a reproducibility of 100%.

Statistical analysis

Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) 11.5 software package (SPSS Company, Chicago, IL, USA). Hardy–Weinberg equilibrium analysis was performed to compare the
observed and expected genotype frequencies using the χ² test. Comparisons of the FGF1 -1385A/G and FGF2 754C/G genotype and allele distributions and their combined effect in the study groups were performed by means of two-sided contingency tables using the χ² test. The OR and 95% CI were calculated using an unconditional logistic regression model. A probability level of 5% was considered significant for all statistical analyses.

### Results

#### General characteristics of the study subjects

The mean age of EM patients and controls was 35 years (range 16–51 years) and 36 years (range 16–51 years), respectively; the mean age of adenomyosis patients and controls was 42 years (range 16–55 years) and 42 years (range 16–55 years), respectively. There was no significant difference in age distribution between the cases and controls in the two groups. The frequency distributions of the FGF1 -1385A/G and FGF2 754C/G genotypes in both control groups did not significantly deviate from that expected for Hardy–Weinberg equilibrium ($\chi^2 = 0.94, 2.60$ and $P = 0.33, 0.11$, respectively, for EM; $\chi^2 = 3.25, 2.80$ and $P = 0.07, 0.09$, respectively, for adenomyosis).

#### Association of FGF1 -1385A/G and FGF2 754C/G with the risk of EM

Genotype frequencies of FGF1 -1385A/A, A/G and G/G in the cases were 7.8, 36.3, 55.8%, and 10.9, 41.1, 48.0% in the controls, respectively. There was no difference between the two groups ($P = 0.053$), but the frequency of the A allele in the EM patients was significantly lower than that of control women ($P = 0.013$) (Table I). Compared with the G/G genotype, the A/G + A/A genotype was associated with the risk of developing EM (OR = 0.744, 95% CI = 0.564–0.981) (Table II).

For the FGF2 754C/G polymorphism, the genotype and allele frequencies in the cases were significantly different from those in the controls ($P = 0.005$ and $P = 0.003$) (Table I). Compared with the C/C genotype, carriers of the G allele had a significantly reduced risk of EM (OR = 0.575, 95% CI = 0.387–0.854) (Table II).

#### Association of FGF1 -1385A/G and FGF2 754C/G with the risk of adenomyosis

There was no significant difference in the frequency distributions of the genotypes and alleles of the FGF1 -1385A/G polymorphism between the cases and controls (Table I). Compared with the G/G genotype, the A/G and A/A genotypes did not influence the risk of developing adenomyosis (Table II).

The genotype and allele frequency distributions of the FGF2 754C/G polymorphism in the cases did show significant differences from those of the controls ($P = 0.019$ and $P = 0.021$) (Table I). Compared with the FGF2 754C/C genotype, the CG + GG genotype

### Table I The genotype and allele frequency distributions for the two FGF polymorphisms in north Chinese cases and controls.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Controls n (%)</th>
<th>EM n (%)</th>
<th>P-value</th>
<th>Controls n (%)</th>
<th>Adenomyosis n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>FGF1 -1385G/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>421(100)</td>
<td>421(100)</td>
<td>0.053</td>
<td>269(100)</td>
<td>269(100)</td>
<td>0.277</td>
</tr>
<tr>
<td>G/G</td>
<td>202(48.0)</td>
<td>235(55.8)</td>
<td></td>
<td>127(47.2)</td>
<td>142(52.8)</td>
<td></td>
</tr>
<tr>
<td>G/A</td>
<td>173(41.1)</td>
<td>153(36.3)</td>
<td></td>
<td>106(39.4)</td>
<td>101(37.5)</td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>46(10.9)</td>
<td>33(7.8)</td>
<td></td>
<td>36(13.4)</td>
<td>26(9.7)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>577(68.5)</td>
<td>623(74.0)</td>
<td>0.013</td>
<td>360(66.9)</td>
<td>385(71.6)</td>
<td>0.099</td>
</tr>
<tr>
<td>A</td>
<td>265(31.5)</td>
<td>219(26.0)</td>
<td></td>
<td>178(33.1)</td>
<td>153(28.4)</td>
<td></td>
</tr>
<tr>
<td>FGF2 754C/G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>421(100)</td>
<td>421(100)</td>
<td>0.005</td>
<td>269(100)</td>
<td>269(100)</td>
<td>0.019</td>
</tr>
<tr>
<td>C/C</td>
<td>345(81.9)</td>
<td>374(88.8)</td>
<td></td>
<td>210(78.1)</td>
<td>231(85.9)</td>
<td></td>
</tr>
<tr>
<td>C/G</td>
<td>69(16.4)</td>
<td>44(10.5)</td>
<td></td>
<td>52(19.3)</td>
<td>33(12.3)</td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>7(1.7)</td>
<td>3(0.7)</td>
<td></td>
<td>7(2.6)</td>
<td>5(1.9)</td>
<td></td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>759(90.1)</td>
<td>792(94.1)</td>
<td>0.003</td>
<td>472(87.7)</td>
<td>495(92.0)</td>
<td>0.021</td>
</tr>
<tr>
<td>G</td>
<td>83(9.9)</td>
<td>50(5.9)</td>
<td></td>
<td>66(12.3)</td>
<td>43(8.0)</td>
<td></td>
</tr>
</tbody>
</table>
appeared to significantly decrease the risk of developing adenomyosis (OR = 0.577, 95% CI = 0.367–0.906) (Table II).

### Combined genotype analysis of the FGF1 and FGF2 polymorphisms on the risk of EM and adenomyosis

As shown in Table III, combination analysis of the FGF1 -1385A/G and FGF2 754C/G SNPs showed that there was a significant difference between the two case–control groups (all \( P < 0.001 \)). Compared with the wild type GG/CC, the AA/GG, AA/CG, GA/GG and GA/CG significantly modified the risk of developing EM (OR = 0.610, 95% CI = 0.393–0.896; OR = 0.601, 95% CI = 0.441–0.821; OR = 0.778, 95% CI = 0.610–0.993; OR = 0.731, 95% CI = 0.586–0.912, respectively); the AA/CG significantly decreased the risk for adenomyosis development (OR = 0.606, 95% CI = 0.422–0.869).

### Discussion

Our results show that genetic variations of the 754C/G at intron I in the FGF2 gene were associated with the risk of EM and adenomyosis development in women of northern China, i.e. carrying the 754G allele may significantly decrease the risk of developing EM and adenomyosis. To the best of our knowledge, this is the first study to look for an association between polymorphisms in the FGF1 and FGF2 gene and the risk of developing EM and adenomyosis.

The FGF1 gene is localized on chromosome 5q31.3–33.2 (Jay et al., 1986; Huebner et al., 1990), spanning more than 100 kb and containing three protein-coding exons (Payson et al., 1998). Transcription of the FGF1 gene is controlled in a tissue-specific manner by at least four distinct promoters: 1.A and 1.B, respectively, active in the kidney and brain, and 1.C and 1.D in a variety of cells, including the vascular smooth muscle cells (Myers et al., 1993; Chiu et al., 2001). The DNA fragment containing 1614 nucleotides upstream of the FGF-1.C transcription start site is sufficient for stimulating the 1.C promoter (Payson et al., 1998). Hence, it is reasonable (Yamagata et al., 2004) to speculate that the -1385A/G polymorphism could influence the FGF-1.C promoter activity.

Although the mechanism of how the FGF1 -1385A/G polymorphism may regulate gene expression is unclear, a previous study showed that this promoter SNP was associated with the risk of late-onset Alzheimer’s disease (LOAD) (Yamagata et al., 2004); compared with the control subjects, the frequency of the FGF1 -1385G
allele was significantly increased in the LOAD patients of a Japanese population, and the GG homozygotes had a higher risk of LOAD than those carrying the A allele. However, Butt et al. (2007) did not observe an association between the FGF1 -1385A/G polymorphism and psoriatic arthritis. In the present study, although the genotype frequency of FGF1 -1385A/G was not significantly different between EM patients and control women, the frequency of the G allele was significantly higher in the EM patients. Therefore, the carriers of the A allele may have significantly decreased risk of developing EM in this population.

FGF2, as another important member of the FGF family, is located on chromosome 4q26–27, and the primary translation product consists of 155 amino acids (Mergia et al., 1986). This gene comprises three exons, separated by two introns and large 5′- and 3′-untranslated regions (Abraham et al., 1986). FGF2 is one of the most potent angiogenic growth factors reported so far (Goto et al., 1995) and can up-regulate the expression of VEGF and synergistically act with VEGF in stimulating new vessel formation (Pepper et al., 1992; Stavri et al., 1995; Seghezzi et al., 1998). FGF2 754C/G may be a functional SNP even though it lies in intron 1. Beranek et al. (2008) reported that there was a trend for different genotype frequencies of the 754C/G polymorphism between PDR and non-diabetic groups (P = 0.05), but the plasma level of FGF2 protein in GG homozygotes (median plasma level of FGF2 is 33.1 pg/ml) was significantly lower than that in carriers of the C allele (69.4 pg/ml) in the PDR patients. Furthermore, they presumed that the FGF2 754C/G polymorphism may cause a structural change in the FGF2 mRNA, which could play a role in post-transcriptional modifications.

Our study shows that the genotype and allele frequencies of the FGF2 754C/G polymorphism in two case groups are significantly different from those in two control groups. The frequency of the G allele in patients was lower than that of control women and thus, carriers of the G allele may have significantly decreased risk of developing EM and adenomyosis. These results suggest that the FGF2 754C/G polymorphism may be a marker associated with decreased penetrance of these two gynecologic diseases.

EM and adenomyosis are regarded as complex traits, in which genetic and environmental factors act together to produce the phenotypes. So far, most studies of candidate genes are generally chosen based on biological mechanisms. There is evidence suggesting familial aggregation of EM and genetic linkage to chromosomes 7 and 10 (Trelaar et al., 2005), but the genes (or variants) in these regions contributing to disease risk have yet to be identified. The FGF1 and FGF2 genes are located at chromosomes 5q and 4q. Considering these two genes are implicated in angiogenesis, which is crucial in the pathophysiology of EM and adenomyosis (Healy et al., 1998; Laschke and Menger, 2007), we hypothesized that polymorphisms in these genes may be related to susceptibility of EM and adenomyosis: the results of the present study have supported our view.

Although the sample size of present study is not large, the result may be reliable considering the following aspects: (i) all the cases in this research are surgically diagnosed with histological confirmation of disease, (ii) the frequency distributions of two SNP genotypes in the control groups did not significantly deviate from that expected for Hardy–Weinberg equilibrium, and the allele frequencies are similar to that in The National Center for Biotechnology Information database and (iii) the association between the FGF2 754C/G polymorphism and the risk of EM and adenomyosis was confirmed in two independent case–control studies. In our study, some controls were undergoing physical examination at the hospital, therefore it is possible that some preclinical cases of EM and adenomyosis may be present in the control group, but this is unlikely to affect the conclusion from this study for EM, with a prevalence of 8–10% (Moskvina et al., 2005).

In conclusion, our study in women from north China found for the first time that the FGF2 754C/G polymorphism may be associated with a risk of developing EM and adenomyosis. The G allele in the FGF2 gene may potentially protect against the development of EM and adenomyosis in this particular population. Further studies in other populations and ethnic groups, as well as functional evaluation of the transcript variants, are warranted to confirm our findings. Given the complexities of both EM and adenomyosis, other SNPs in genes associated with angiogenesis and/or other cellular pathways also need to be investigated.

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Fibroblast growth factor polymorphism and endometriosis


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