Reproductive genetics

Ovarian cancer-associated polymorphisms in the BNC2 gene among women with endometriosis

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Background: Endometriosis is a common benign gynaecological disease. Epidemiological studies have demonstrated associations between endometriosis and ovarian cancer. Recent genome-wide association studies of ovarian cancer have identified several single nucleotide polymorphisms (SNPs) in the Basonuclin 2 (BNC2) gene. In this study, we investigated these polymorphisms in women with endometriosis.

Methods: Six SNPs in and upstream of the BNC2 gene (rs3814113, rs4445329, rs10962656, rs12379183, rs10756819 and rs1339552) were investigated using TaqMan allelic discrimination analysis in a Caucasian population (cases: 798, controls: 351). Allelic frequencies were used as main outcome measure.

Results: No associations were observed between the analysed SNPs and endometriosis.

Conclusions: Our results suggest that the analysed polymorphisms in the BNC2 gene are unlikely to contribute to the previously reported risk of ovarian cancer in women with endometriosis.

Key words: endometriosis / ovarian cancer / single-nucleotide polymorphism / BNC2

Introduction

Endometriosis mainly affects women of reproductive age and is characterized by a wide clinical panorama. Apart from symptoms related to the pelvic presentation of the disease, such as dysmenorrhea and subfertility, studies show an increased incidence of depression, reduced quality of life (Jones et al., 2004; Lorenzatto et al., 2006) and major health-care costs (Simoens et al., 2007). In spite of recent advances in endometriosis research, the etiology remains unclear. Several reports suggest that the disease is inherited as a complex genetic trait (Kennedy, 1999; Kennedy et al., 2001), involving multiple genes together with environmental factors (Bischoff and Simpson, 2000; Falconer et al., 2007).

Although endometriosis is considered a benign condition, the disease demonstrates malignant characteristics (Varma et al., 2004; Nezhat et al., 2008). Endometriosis involves loss of control of cell proliferation, tissue invasion with both local and distant spread and a chronic inflammatory state. Genetic, angiogenic, endocrine and immunological abnormalities have been observed in endometriosis patients, compared with healthy women (Erzen and Kovacic, 1998; Modesitt et al., 2002). It has also been suggested that endometriotic implants can directly undergo malignant transformation, or that endometriosis and ovarian cancer share common mechanisms and/or predisposing factors, such as genetic susceptibility or immune and angiogenic dysregulation (Varma et al., 2004). However, the association between ovarian cancer and endometriosis is debated (Brinton et al., 1997; Melin et al., 2006; Somigliana et al., 2006; Gemmill et al., 2010). Data from large cohort and case–control studies indicate an increased risk of ovarian cancer in women with endometriosis, although the observed effect sizes are modest varying between...
1.3 and 1.9 (Brinton et al., 1997; Melin et al., 2006; Somigliana et al., 2006; Gemmill et al., 2010). Melin et al. (2006) reported an increased risk for endometriosis patients of developing not only ovarian cancer, but also endocrine tumors, non-Hodgkin’s lymphoma and brain tumors, with a more pronounced risk in women with early diagnosed and long-standing endometriosis. These results are in line with those from retrospective studies showing an increased risk of ovarian cancer in women with ovarian endometriosis (Erzen and Kovic, 1998; Stern et al., 2001). The evidence from clinical series consistently demonstrates that the association is confined to the endometrioid/clear-cell histotypes of ovarian cancer (Fukunaga et al., 1997; Ogawa et al., 2000).

To date, no data exist to inform clinicians which patients with endometriosis are at risk of developing ovarian cancer (Kennedy et al., 2005) and prophylactic surgical procedures are questionable for this group of women (Somigliana et al., 2006). In view of the severe nature of ovarian cancer, a search for contributing/protective genetic markers (SNPs) of the disease is justified in women with endometriosis, even if the effect sizes of an association are probably small.

In ovarian cancer, specific genes involved in many important pathways, including cell cycle regulation (Goode et al., 2009; Quaye et al., 2009), DNA repair and steroid hormone pathways (Fasching et al., 2009) have been implicated in the pathogenesis. In a recent genome-wide association study (GWAS), an ovarian cancer associated locus has been identified on chromosome 9p22.2 and strong associations were observed for 12 single-nucleotide polymorphisms (SNPs) in the Basonuclin 2 (BNC2) gene (Song et al., 2009). Also, gene expression of BNC2 in ovarian cancer cell lines is significantly lower compared with that in normal primary human ovarian surface epithelial cell cultures (Goode et al., 2010). However, the identified SNPs were associated with a decreased risk of ovarian cancer, which may indicate that susceptibility for ovarian cancer is driven by a single, correlated variant within the region. In this study, we tested the hypothesis that SNPs in and upstream of the BNC2 gene are associated with endometriosis in a Caucasian population. Furthermore, in a sub-analysis, differences in the distribution of BNC2 SNPs between stage I/II and III/IV disease were analysed. The selection of six SNPs was based on position of the SNP in the BNC2 gene, linkage disequilibrium and available assays.

**Materials and Methods**

**Clinical material**

A total of 1049 women (798 endometriosis patients and 351 controls) who had undergone a laparoscopy for subfertility with or without pain at the Leuven University Hospital, Leuven, Belgium during 1998–2007 were included in our study based on the following inclusion criteria: Caucasian ethnicity, laparoscopic and histologically proven presence of endometriosis or laparoscopic absence of endometriosis according to electronic medical file records, DNA material or sources available. Women with endometriosis (age: 31 ± 4 years; BMI: 23.2 ± 4) had either minimal (stage I; n = 176), mild (stage II; n = 116), moderate (stage III; n = 88) or severe (stage IV; n = 179) disease, staged according to the revised classification system of the American Society for Reproductive Medicine (1997). The absence of endometriosis was confirmed laparoscopically in control patients (age: 32 ± 5 years; BMI: 23.4 ± 4). Patient characteristics are summarized in Table I.

**DNA extraction**

DNA purified from EDTA stabilized whole peripheral blood was collected for routine molecular diagnostic tests at the Center for Medical Genetics of University Hospitals, Leuven, Belgium. Various purification methods were used: Chemagic DNA blood special kit (Chemagen, Baesweiler, Germany) based on the specific binding of DNA to paramagnetic beads, Auto Pure LS Puregene chemistry (Qiagen, Venlo, The Netherlands) based on salting-out extraction and a manual salting-out procedure (home-brew). The choice of the extraction method was based on the available amount of blood and on the type of required molecular diagnostic test. DNA from peritoneal biopsies was purified with QIAGEN QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. DNA concentration was measured with NanoDrop 1000 (Thermo Scientific, Wilmington, USA). For tissue samples with 260:280 ratios <1.5, a DNA clean-up/purification with phenol–chloroform (P 2069, Sigma-Aldrich, Saint Louis, Missouri, USA) was performed. An equal volume of phenol–chloroform was added to the sample, mixed and centrifuged. The aqueous phase was collected. The procedure was repeated with chloroform, followed by ethanol precipitation, where double the sample volume of ice cold 99.5% ethanol was added. After centrifugation, ethanol was removed and the pellet was left to dry before being resuspended for 2 h in 1 × Tris–EDTA-buffer pH 8.0 in 37°C.

**SNP genotyping**

DNA samples were diluted to a final concentration of ~2 ng/μl in 96-well plates. Blanks and duplicates were randomly distributed on all 96-well plates in order to estimate the quality of the samples. 384-well plates with DNA were prepared by a Biomek FX (Beckman Coulter, Fullerton, USA) with 10 ng DNA/well. TaqMan allelic discrimination analyses were performed according to Applied Biosystems standard protocols (Applied Biosystems, Carlsbad, USA). The analysed SNPs were as follows: rs4445329 (C_70469_10).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Endometriosis</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>798</td>
<td>351</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Caucasian</td>
<td>Caucasian</td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>31 ± 4</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.2 ± 4</td>
<td>23.4 ± 4</td>
</tr>
<tr>
<td>Smoking, yes (%)</td>
<td>18.7</td>
<td>21.2</td>
</tr>
<tr>
<td>Stage I (n)</td>
<td>176 (22.05%)</td>
<td>NA</td>
</tr>
<tr>
<td>Stage II (n)</td>
<td>116 (14.54%)</td>
<td>NA</td>
</tr>
<tr>
<td>Stage III (n)</td>
<td>88 (11.03%)</td>
<td>NA</td>
</tr>
<tr>
<td>Stage IV (n)</td>
<td>179 (22.43%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Peripheral blood samples (n = 948; 660 endometriosis patients and 288 controls with laparoscopically excluded endometriosis) and peritoneal biopsies taken during surgery (n = 229; 161 endometriosis patients and 68 controls) were collected. From some patients and controls (n = 28) both blood sample and tissue were obtained. The study was approved by the Commission for Medical Ethics of the Leuven University Hospital Belgium and the Regional Ethics committee, Karolinska Institute (KI), Stockholm, Sweden.

**Table I Characteristics of study population.**

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rs10962656 (C_31294047_10), rs12379183 (C_36020_10), rs10756819 (C_69643_10) and rs1339552 (C_742906_20) (Applied Biosystems), and rs3814113 for which a custom made Taqman assay (Applied Biosystems) was made, using primers and probes (forward primer CCAAACCC CTCCCTGATACG, reverse primer GCCCAGCCAGTTTGGAGAA, VIC probe CCTGCTCCATATCTTCTGGA and FAM probe CTGCTCCA- TATCTCCTGGA) according to Song et al. (2009). PCR was performed according to following amplification protocol: denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s. and then annealing and extension at 60 °C for 1 min. Plates were read in a 7900 HT Fast-Real-time PCR system (Applied Biosystems) using the program SDS 2.2 (Applied Biosystems).

Statistics
As quality control, Hardy–Weinberg equilibrium (HWE) and call rate was calculated. SNP associations were analyzed using the allelic model with odds ratio (OR) and 95% confidence intervals. Corrections were made using the Bonferroni correction, in which the obtained P-values were multiplied by the number of markers and analysis (×12). P-values <0.05 were considered significant. Haplotype analysis was performed using Haploview 4.2 (Cambridge, MA, USA).

Results
Association of BNC2 with endometriosis
Taqman runs for rs4445329, rs12379183, rs10756819 and rs1339552 where of high quality, with a call rate >95%, and rs3814113 and rs10962656 had a call rate >92%. None of the SNPs had a significant Hardy–Weinberg level (HW <0.05). The minor allele frequency (MAF) in the control population corresponded well to the MAF found in the HapMap database (http://www.hapmap.org) (data not shown). The result from the analyses of the allelic model, using the minor allele in the calculations, is summarized in Table II. No association of BNC2 rs3814113, rs4445329, rs10962656, rs12379183, rs10756819 or rs1339552 with endometriosis was found. Also, haplotype analysis showed no significant association (data not shown).

Association of BNC2 with different stages of endometriosis
The endometriosis group was divided into two groups depending on the stage of the disease: stage I and II (minimal/mild disease) and stage III and IV (moderate/severe disease). None of the investigated SNPs showed association with the two groups before, as shown in Table III, or after Bonferroni correction (data not shown).

Discussion
Endometriosis has been indicated as a potential risk factor for endometrioid/clear-cell ovarian cancer. However, the underlying molecular and genetic background for this association is unknown. Several genetic polymorphisms have been implicated in the pathogenesis of ovarian cancer (Notariou et al., 2011). In one of the most recent GWAS conducted in ovarian cancer, a strong association with BNC2 was found, where the minor allele of the associated SNPs were protective against disease (Song et al., 2009). In spite of a large sample set, we were unable to demonstrate any association of the six selected BNC2 polymorphisms with endometriosis, although there was a very small tendency towards a higher frequency of the associated minor allele in controls. Although we have reported differences in genetic association (HLA-DRB1 and CCL21) between minimal/mild and moderate/severe endometriosis (Sundqvist et al., 2011), the sub-analysis in this study did not show any differences in the distribution of selected BNC2 SNPs between the different stages of the disease. GWAS conducted in endometriosis have not reported any associations of these SNPs and endometriosis (Adachi et al., 2010; Painter et al., 2010; Uno et al., 2010), which is in agreement with our results. We conclude that it is unlikely that the analysed polymorphisms in the BNC2 gene are associated with endometriosis.

The function of BNC2 is unclear. It consists of three pairs of zinc fingers, a nuclear localization signal and shares evolutionary origin with Basonucin 1 (BNC1) (Vanhoutteghem and Djian, 2006). However, these two proteins have different properties and functions. The function of BNC1 relates to cell proliferation, while BNC2 co-localizes with SC35, a splicing factor, in keratinocytes and is believed to participate in mRNA processing (Vanhoutteghem and Djian, 2006). Romano et al. (2004) also showed binding of BNC2 to the rRNA promoter. In addition, BNC2 seems to have an extensive transcriptional variability, containing six promoters, giving rise to 90 000 potential mRNA isoforms encoding more than 2000 proteins (Vanhoutteghem and Djian, 2007). Reproductive tissues, such as ovary, uterus and testis, show expression of BNC2 (Romano et al., 2004; Vanhoutteghem and Djian, 2004), although the precise function of BNC2 in these tissues are unclear.

Endometriosis is a complex disease with multiple interacting etiological mechanisms and the effect size of each individual factor is most likely small. The impact of genetic changes is for the most part unknown, although several attempts have been made to elucidate the role of SNPs in endometriosis. It could be argued that the majority of association studies fail to delineate the relationship between complex disease and certain SNPs. A marginal risk for disease development combined with a presumably small effect size of an individual SNP creates a situation where the results are cumbersome to interpret. In contrast to many other complex diseases, such as diabetes and rheumatoid arthritis, few GWAS have been conducted in endometriosis (Adachi et al., 2010; Painter et al., 2010; Uno et al., 2010). These studies are highly complex and require vast economic and technological resources. Collaterally with the development of advanced GWAS, well-designed association studies are still a viable option (Montgomery et al., 2008). The failure of many association studies in endometriosis can largely be attributed to poorly defined hypotheses and small sample sizes (Falconer et al., 2007). Many of these studies have analyzed disease populations of less than 200 cases, a situation most likely caused by the invasive nature of endometriosis diagnosis. Selecting candidate genes for endometriosis from GWAS in related areas like ovarian cancer creates a solid foundation for genetic analysis studies. As it was reported in a GWAS with 1817 cases of ovarian cancer that the strongest association with BNC2 polymorphisms conferred a 20% reduction in risk with each copy of the minor allele (Song et al., 2009), we investigated these polymorphisms in 1149 women with and without endometriosis. In this study, we observed a difference in MAF distribution of about 7% between cases and controls. It should be noted that although our sample size was large compared with most other association studies (Falconer et al., 2007), studies of complex diseases (such as endometriosis)
generally require even larger populations to detect reasonably common SNPs. However, with such a small difference in MAF, we find it unlikely that a larger study population would change the results significantly.

In conclusion, we were unable to find any association between ovarian cancer-related polymorphisms in the BNC2 gene and endometriosis. The process of ovarian cancer development in women with endometriosis is probably mediated by different pathways. Other candidate genes, involved in both endometriosis and ovarian cancer, may be of interest for future investigation.

**Authors’ roles**

J.S., H.F., M.S., K.G.D. contributed to the design of the study. J.S., A.V., A.F., C.K., A.B., O.S. and T.M.D. were involved in sample collection and performance of the study. Acquisition of data and analysis were done by J.S. and M.S. Manuscript writing was performed by J.S. and H.F. Reviewing of the manuscript was done by J.S., H.F., M.S., A.V., A.F., C.K., A.B., O.S., K.G.D. and T.M.D.

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**Table II** Genotype frequencies of BNC2 polymorphisms in endometriosis patients and controls.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>Genotype frequencies, controls/patients</th>
<th>Allelic model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/1</td>
<td>1/2</td>
</tr>
<tr>
<td>Rs3814113 (C/T)*</td>
<td>≏ 44 kb upstream</td>
<td>0.134/0.110</td>
<td>0.404/0.398</td>
</tr>
<tr>
<td>Rs4445329 (A/G)*</td>
<td>≏ 41 kb upstream</td>
<td>0.123/0.104</td>
<td>0.419/0.418</td>
</tr>
<tr>
<td>Rs10962656 (A/G)*</td>
<td>7 kb upstream</td>
<td>0.028/0.020</td>
<td>0.279/0.257</td>
</tr>
<tr>
<td>Rs12379183 (G/A)*</td>
<td>Intron 2</td>
<td>0.042/0.041</td>
<td>0.374/0.393</td>
</tr>
<tr>
<td>Rs10756819 (G/A)*</td>
<td>Intron 2</td>
<td>0.119/0.100</td>
<td>0.436/0.448</td>
</tr>
<tr>
<td>Rs1339552 (T/C)*</td>
<td>Intron 2</td>
<td>0.200/0.170</td>
<td>0.479/0.484</td>
</tr>
</tbody>
</table>

CI, confidence interval; OR, odds ratio.

*The minor allele in controls is given first.

**Table III** Genotype frequencies of BNC2 polymorphisms in patients, divided into minimal/mild and moderate/severe disease, compared with controls.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Disease status</th>
<th>Genotype frequencies, controls/patients</th>
<th>Allelic model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/1</td>
<td>1/2</td>
</tr>
<tr>
<td>Rs3814113 (C/T)*</td>
<td>Minimal/mild</td>
<td>0.134/0.130</td>
<td>0.404/0.413</td>
</tr>
<tr>
<td></td>
<td>Moderate/severe</td>
<td>0.134/0.102</td>
<td>0.404/0.380</td>
</tr>
<tr>
<td>Rs4445329 (A/G)*</td>
<td>Minimal/mild</td>
<td>0.123/0.123</td>
<td>0.419/0.437</td>
</tr>
<tr>
<td></td>
<td>Moderate/severe</td>
<td>0.123/0.093</td>
<td>0.419/0.413</td>
</tr>
<tr>
<td>Rs10962656 (A/G)*</td>
<td>Minimal/mild</td>
<td>0.028/0.026</td>
<td>0.279/0.273</td>
</tr>
<tr>
<td></td>
<td>Moderate/severe</td>
<td>0.028/0.012</td>
<td>0.279/0.262</td>
</tr>
<tr>
<td>Rs12379183 (G/A)*</td>
<td>Minimal/mild</td>
<td>0.042/0.032</td>
<td>0.374/0.418</td>
</tr>
<tr>
<td></td>
<td>Moderate/severe</td>
<td>0.042/0.054</td>
<td>0.374/0.366</td>
</tr>
<tr>
<td>Rs10756819 (G/A)*</td>
<td>Minimal/mild</td>
<td>0.119/0.091</td>
<td>0.436/0.490</td>
</tr>
<tr>
<td></td>
<td>Moderate/severe</td>
<td>0.119/0.110</td>
<td>0.436/0.421</td>
</tr>
<tr>
<td>Rs1339552 (T/C)*</td>
<td>Minimal/mild</td>
<td>0.200/0.187</td>
<td>0.479/0.489</td>
</tr>
<tr>
<td></td>
<td>Moderate/severe</td>
<td>0.200/0.155</td>
<td>0.479/0.490</td>
</tr>
</tbody>
</table>

CI, confidence interval; OR, odds ratio.

*The minor allele in controls is given first.

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