Single nucleotide polymorphisms of follicle-stimulating hormone receptor are associated with ovarian cancer susceptibility

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Epidemiological studies suggested that ovulation was associated with ovarian carcinogenesis. Follicle-stimulating hormone (FSH) played an important role in follicular development and was recently found to affect growth of ovarian epithelial cells. Single nucleotide polymorphisms (SNPs) Thr307Ala and Asn680Ser were two non-synonymous variations in the coding region of the FSH receptor (FSHR) gene. This hitherto first case–control study investigating the association between these two FSHR SNPs and the risk of ovarian cancer involved 202 histopathologically confirmed ovarian cancer patients and 266 age-matched cancer-free control subjects using restriction fragment length polymorphism assay and direct sequencing. Our results demonstrated that the 307Ala and 680Ser carriers were associated with significantly increased risk of developing serous and mucinous types of ovarian cancers (P<0.0005, OR = 2.60, 95% CI = 1.56–4.34; and P<0.0005, OR = 2.89, 95% CI = 1.73–4.84, adjusted for age, respectively) but not endometrioid and clear cell types. The two SNPs were found to be in modest linkage disequilibrium, D’ = 0.804 and 0.701, r² = 0.581 and 0.406 for the cancer and control groups, respectively. The major haplotype of 307Ala-680Ser was also associated with higher cancer risk (P = 0.033, OR = 1.39, 95% CI = 1.03–1.88), especially for the serous and mucinous carcinomas (P = 0.001, OR = 1.82, 95% CI = 1.27–2.60). Our results suggested that the two FSHR SNPs might affect the susceptibility of women to specific subtypes of ovarian cancer. Different types of ovarian cancer might adopt distinct carcinogenic pathways. Such understanding may be important in selecting patients for ovulation induction therapy.

Introduction

Ovarian cancer contributes to the highest mortality among all gynecological cancers and difference in incidence of ovarian cancer in different ethnic groups was reported (1). Important hypotheses regarding ovarian carcinogenesis include stimulation due to incessant ovulation, gonadotropins and sex-steroid hormones (2). Epidemiological studies suggested that events that would increase the number of ovulation such as pregnancy, oral contraceptive use and breastfeeding would significantly elevate the risk of developing ovarian cancer (3). Ovulation was implicated as a risk factor for ovarian cancer (4,5).

Follicle-stimulating hormone (FSH) is essential for ovarian follicular development. FSH evokes its biological effects by interacting with high affinity receptor located on the plasma membrane of its target cells in the gonads. FSHR SNPs also suggested that different haplotypes were significantly related to different basal level of serum FSH (17). As a result, amino acid alteration related to the corresponding SNPs might affect the post-translational modifications of the FSHR protein, and hence the function of the receptor including FSH efficacy (18). Haplotype analysis on these two FSHR non-synonymous SNPs also suggested that different haplotypes were significantly related to different basal level of serum FSH (19). Such observations have promoted our interest in investigating the role of FSHR polymorphism in ovarian carcinogenesis.

In this study, we postulated that two non-synonymous SNPs of the FSHR gene, Ala307Thr and Ser680Asn located at the nucleotide positions of 919 and 2039 of the FSHR coding sequence (GeneBank accession no. NM_000145), may affect the susceptibility of ovarian epithelium to development of cancer.

Materials and methods

Study population and data

Archival paraffin embedded tissue blocks were retrieved from the files (1985–2002) of the Department of Pathology, Queen Mary Hospital, a major referral center in Hong Kong for patients with gynecologic...
malignancies. Hematoxylin and eosin-stained section of each tissue blocks was assessed to review the diagnosis and ensure the absence of tumor before performed DNA extraction. Two hundred and twenty ovarian cancer cases with available paraffin embedded non-tumor tissue were retrieved with success. DNA extraction performed in 202 cases. The mean age of these 202 cancer patients at diagnosis was 51.40 ± 12.6 years (range 23–83 years). The histological subtypes, which included serous (SC, n = 68), mucinous (MC, n = 36), endometrioid (EC, n = 68) and clear cell carcinoma (CC, n = 30), were reviewed by histopathologists. Ovarian borderline tumors, sex-cord stromal tumors, germ cell tumors and metastatic tumors were excluded. The mean ages for serous, mucinous, endometrioid and clear cell types of cancer cases were similar: 54.7, 50.1, 49.6 and 49.8, respectively. Two hundred and sixty-six randomly selected control subjects who had undergone salpingo-oophorectomy for benign conditions and known not to have ovarian carcinoma were included in this study. Their paraffin embedded tissues were retrieved from the Department of Pathology, Queen Mary Hospital. The mean age of control subjects was 49.26 ± 11.60 years (range 20–81 years). These control subjects were diagnosed to have leiomyomas (n = 238), tubal ectopic pregnancy (n = 6), endometrial polyp (n = 2) and uterine prolapse (n = 20). All of the studied cancer and control subjects were of Chinese origin, while three of the cancer patients were born in Vietnam and Philippines. Out of the 202 cancer patients, data regarding previous hormone replacement, ovulation or contraceptive drug history was available in 177 patients. Six have received birth control pills while one has received hormonal replacement therapy.

Extraction of DNA

Microdissection was performed if necessary so that only tissue without tumor contamination was used for DNA extraction. Ten consecutive 10 μm sections were cut from each paraffin embedded tissue block. Genomic DNA was then extracted from the deparaffinized tissue using the conventional phenol/ chloroform method following the proteinase K digestion (20). DNA was then ethanol precipitated, vacuum dried and then suspended in 1x TE buffer.

Genotyping

The two SNPs, Ala307Thr and Ser680Asn (rs6165 and rs6166 in dbSNP, respectively), introduced restriction sites that could be investigated using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique (13,19,21) with the primers which were designed based on the published sequence of the human FSHR gene (GeneBank accession no. NM_000145): forward 5′-GCT AGC TTC ATC CAA TTG G3′ and reverse 5′-CTC TGC TGT AGC TGG ACT CAT T-3′ for Ala307Thr, forward 5′-CCC AAA TTT ATA GGA CAG-3′ and reverse 5′-GAG GGA CAA GTA TGT AAG TG-3′ for Ser680Asn. The PCR was carried out in 20 μl containing 1x PCR buffer, 3 mM MgCl2, 200 mM dNTP and 0.6 μl of AmpliTaq polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 300 nM each forward and reverse primers. The reaction mixtures were heated to 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 55°C (for Ala307Thr) or 50°C (for Ser680Asn) for 30 s, and 72°C for 30 s with final extension of 72°C for 5 min. The amplified PCR products (120 and 114 bp, respectively) were digested by restriction enzymes of AhdI (for SNP Ala307Thr) and BsrI (for SNP Ser680Asn) (New England Biolabs Inc., Beverly, MA, USA) at the optimized conditions for 18 h and then separated by 8% polyacrylamide gel electrophoresis (13,19). After electrophoresis, the gel was stained by ethidium bromide and then visualized under UV. In each run of PCR and digestion, control samples with known homozygous and heterozygous genotypes (confirmed by direct sequencing) of these two SNPs were included. The known homozygous and heterozygous genotype controls were also included in each set of restriction enzyme digestion to ensure complete digestion. Five percent of cases were randomly selected and subjected to direct sequencing to confirm the PCR–RFLP findings. Twenty percent of the total studied cases were repeated in this genotyping experiment. Negative control with no DNA template was included in each run of PCR. The genotype results were scored by two individuals separately.

Statistical analysis

χ2-test was used to evaluate the association of the FSHR genotypes in the case–control populations. Odds ratio (OR) and 95% confidence interval (CI) were used to measure the strength of the association, where applicable. Logistic regression analysis was used to adjust statistical finding for age. Clinical data, such as histological subtypes, tumor stages and grades of the cancer, had also been analyzed independently for their risk association with the FSHR SNPs. All statistical tests above were two-sided and were performed using the SPSS software (Version 11.0) (SPSS Inc., Chicago, IL, USA). The probability of Hardy–Weinberg equilibrium (HWE) and haplotype frequency estimation (by Expectation and Maximization, EM, algorithm) were assessed using a genetic statistic program, Arlequin Ver. 2.00 (22). Linkage disequilibrium analysis among the SNPs was completed to obtain the linkage disequilibrium coefficient, D′, and the correlation coefficient, r2.

Results

The sizes of amplified products for the SNPs Ala307Thr and Ser680Asn were 120 and 114 bp, respectively. For the SNP Ala307Thr, after digestion by AhdI, three fragments, 70, 31 and 19 bp (Figure 1A), were produced for the Thr/Thr genotype while two fragments, 101 and 19 bp (Figure 1A) were produced for the Ala/Ala genotype. The amplified product harbored one more restriction site for the enzyme resulting in the 19 bp fragment, and that served as an internal digestion control. For SNP Ser680Asn, after digestion by BsrI, two fragments, 86 and 28 bp (Figure 1B) would stand for the Ser/Ser genotype whereas the Asn/Asn genotype remained as the size of 114 bp. The heterozygote was represented by a combination of the fragments found in either genotype. Twenty percent of the total studied cases were duplicated, all had reproducible results. Five percent of cases from each studied populations were directly sequenced, and the sequencing results confirmed the PCR–RFLP findings (Figure 2). The genotypes of the control subjects were in HWE for both SNPs (χ2-test, df = 1, P = 0.286 and 0.731 for SNPs Ala307Thr and Ser680Asn, respectively) while the genotypes in the cancer group was deviated from HWE (P = 0.028 and 0.001 for SNPs Ala307Thr and Ser680Asn, respectively).

The association between SNPs and development of carcinoma was first assessed as a group. Since the EC and CC carcinomas may have different carcinogenic pathways distinct from MC and SC, owing to their strong association with endometriosis (23), their correlation with these two SNPs were separately analyzed in addition.

The genotype frequencies of the SNP Ala307Thr and Ser680Asn were significantly different between the cancer and control groups (P = 0.023 and 0.010, respectively) (Table I). The 307Ala and 680Ser carriers had higher risk to develop ovarian cancer when compared with the studied controls (P = 0.018, OR = 1.58, 95% CI = 1.08–2.30; and P = 0.004, OR = 1.74, 95% CI = 1.19–2.54, adjusted for age, respectively).

By further stratification, the genotypes of the two SNPs were shown to have significant association with the serous and mucinous subtypes (P = 0.001 and P < 0.0005, respectively) (Table I), while the 307Ala and 680Ser carriers were shown.
to have higher risk association \( (P < 0.0005, \text{OR} = 2.60, 95\% \text{ CI} = 1.56–4.34) \) and \( P < 0.0005, \text{OR} = 2.89, 95\% \text{ CI} = 1.73–4.84 \), adjusted for age, respectively. However, such associations were not significant in endometrioid and clear cell subtypes (all \( P > 0.05 \)) (Table I).

The two FSHR SNPs were modestly in linkage disequilibrium in the cancer and control groups as \( D' = 0.80 \) and \( r^2 = 0.58 \), and \( D' = 0.70 \) and \( r^2 = 0.41 \), respectively (Table II).

307Thr-680Asn and 307Ala-680Ser were the major haplotypes, whereas 307Thr-680Ser and 307Ala-680Asn were the minor haplotypes in both populations (Table II). The haplotype 307Ala-680Ser was shown to be associated with higher risk of ovarian cancer \( (P\text{-value} = 0.033, \text{OR} = 1.39, 95\% \text{ CI} = 1.03–1.88) \), particularly for the serous and mucinous subtypes \( (P\text{-value} = 0.001, \text{OR} = 1.82, 95\% \text{ CI} = 1.27–2.60) \). No correlation with this haplotype was demonstrated in the endometrioid and clear cell types \( (P > 0.05) \) (Table II).

There was no statistical significant association between the genotypes with tumor stage \( (P = 0.50 \) and \( 0.34 \) for SNPs Ala307Thr and Ser680Asn, respectively), ascitic involvement \( (P = 0.719 \) and \( 0.359 \) for SNPs Ala307Thr and Ser680Asn, respectively) and patients' age at diagnosis of the cancer \( (P = 0.64 \) and \( 0.67 \) for SNPs Ala307Thr and Ser680Asn, respectively) (data not shown).

**Discussion**

Recent studies have documented FSHR expression in normal surface epithelium of the ovary and the fallopian tube (9) and at a higher level in ovarian cancers (7–9,24). FSHR overexpression was also found to stimulate proliferation in preneoplastic ovarian epithelial cells (25). It was suggested that FSH may be an important growth-promoting factor in ovarian cancer cells (8,10). Fuller et al. (26) in their study focused on granulosa cell tumor, had studied the SNP Ser680Asn in seven mucinous cystadenocarcinoma. Six heterozygous Ser680Asn and one homozygous 680Ser were found suggesting the tendency for 680Ser carriers to have higher risk of developing this cancer. The number of cases, though too few to draw conclusions, concurred with our findings.

Some SNPs in the FSHR promoter region were recently found to alter FSHR expression \textit{in vitro} through changes in transcription factor binding sites although no correlation

Table I. FSHR SNPs genotypes comparison in the cancer and control groups

<table>
<thead>
<tr>
<th></th>
<th>Cancer</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall</td>
<td>SC + MC</td>
</tr>
<tr>
<td>Ala307Thr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>70</td>
<td>26</td>
</tr>
<tr>
<td>Ala/Thr</td>
<td>110</td>
<td>66</td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>P-values*</td>
<td>0.023</td>
<td>0.001</td>
</tr>
<tr>
<td>HWE test (P-value)</td>
<td>0.028</td>
<td>0.003</td>
</tr>
<tr>
<td>Ala carrier</td>
<td>132</td>
<td>78</td>
</tr>
<tr>
<td>Age adjusted P-values**</td>
<td>0.018</td>
<td><strong>&lt;0.0005</strong></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.58 (1.08–2.30)</td>
<td>2.60 (1.56–4.34)</td>
</tr>
<tr>
<td>Ser680Asn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asn/Asn</td>
<td>73</td>
<td>26</td>
</tr>
<tr>
<td>Ser/Asn</td>
<td>114</td>
<td>69</td>
</tr>
<tr>
<td>Ser/Ser</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>P-values*</td>
<td>0.010</td>
<td><strong>&lt;0.0005</strong></td>
</tr>
<tr>
<td>HWE test (P-value)</td>
<td>0.001</td>
<td><strong>&lt;0.0005</strong></td>
</tr>
<tr>
<td>Ser carrier</td>
<td>129</td>
<td>78</td>
</tr>
<tr>
<td>Age adjusted P-values**</td>
<td>0.004</td>
<td><strong>&lt;0.0005</strong></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td>1.74 (1.19–2.54)</td>
<td>2.89 (1.73–4.84)</td>
</tr>
</tbody>
</table>

SC, MC, EC and CC represented serous, mucinous, endometrioid and clear cell types of ovarian cancers, respectively. Boldface indicates \( p \)-values less than 0.05.

*P-values: cancer cases against the controls; \( \chi^2 \)-test.

**Age adjusted \( P \)-values were calculated by logistic regression. HWE test was done by \( \chi^2 \)-test (df = 1).
with basal FSH serum levels or ovarian response in women undergoing controlled ovarian stimulation for IVF treatment could be detected (27). Another study performed on mouse Sertoli cells also suggested that hypermethylation of some CpG sites in the FSHR promoter was associated with down-regulation of FSHR expression (28). Methylation of these CpG sites would hinder the binding of the transcription factors and repressed FSHR expression.

To our best knowledge, this is the first systematic study on the possible association between ovarian cancer and the FSHR non-synonymous SNPs. Overall association analysis showed that the 307Ala carriers and 680Ser carriers were found to carry significantly higher risk to develop ovarian cancers when compared with the non-307Ala carriers and non-680Ser carriers, respectively. These two SNPs were in modest linkage disequilibrium to each other. Our haplotype analysis results showed that the haplotype 307Ala-680Ser had significantly association with cancer risk when compared with the other haplotypes. A study on Japanese women reported that the haplotype 307Ala-680Ser had significantly higher basal level of serum FSH (19), which might enhance proliferation and malignant transformation of the ovarian epithelium and thus contributed to the higher risk of ovarian cancer. This concurred with our findings regarding risk association with the 307Ala and 680Ser carriers and the haplotype 307Ala-680Ser.

Our results demonstrated that different histological subtypes of ovarian cancer displayed different association patterns with the FSHR SNPs. While the FSHR 307Ala and 680Ser allele carriers were associated with increased risk of developing SC and MC, no significant association was found in endometrioid and clear cell types of ovarian cancers. The higher frequency of 307Ala and 680Ser allele carriers in the serous and mucinous types stratified group might contribute to lack of HWE in the overall cancer group, whereas the endometrioid and clear cell types stratified group was shown to be in HWE. Different histological subtypes of ovarian cancers displayed different association patterns with various reproductive risk factors. It is well known that endometrioid and clear cell carcinomas of ovary are highly associated with endometriosis (29). FSH and its receptor may play distinct roles regarding carcinogenesis of different subtypes of ovarian cancers. However, the genotyping results showed no statistical significant association with other clinical parameters such as tumor stages, grades, ascitic fluid involvement and patients’ age at first diagnosis (data not shown).

Our current FSHR polymorphism study performed in Chinese population of Hong Kong, together with data from other studies, demonstrated differences in genotype and allelic frequencies among different populations and ethnic groups (12,30). The high risk genotypes and haplotype in our group was lower that that of the other ethnic groups. Such genetic profile may affect the susceptibility of women in different populations to ovarian cancers. The genotype frequencies of FSHR may also be useful parameters for quality control of the cancer study. The close proximity of genotype frequencies found in our control when compared with Chinese women in Singapore (30,31) suggested that the population we have selected for the present study resembled the randomized Asian populations.

To conclude, our study demonstrated an association between SNPs of FSHR with susceptibility to ovarian cancer, especially in the serous and mucinous subtypes but not the endometrioid

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### Table II. FSHR SNPs haplotypes comparison in the cancer and control groups

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Overall ovarian cancer</th>
<th>SC</th>
<th>MC</th>
<th>EC</th>
<th>CC</th>
<th>OR (95% CI) P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr-680</td>
<td>42.0% (233)</td>
<td>233</td>
<td>107</td>
<td>77</td>
<td>36</td>
<td>0.77 (0.48-1.24) 0.253</td>
</tr>
<tr>
<td>Thr-680</td>
<td>58.0% (307)</td>
<td>307</td>
<td>107</td>
<td>10</td>
<td>15</td>
<td>0.54 (0.29-1.00) 0.053</td>
</tr>
<tr>
<td>Thr-680</td>
<td>58.0% (307)</td>
<td>307</td>
<td>107</td>
<td>10</td>
<td>15</td>
<td>0.54 (0.29-1.00) 0.053</td>
</tr>
<tr>
<td>Thr-680</td>
<td>58.0% (307)</td>
<td>307</td>
<td>107</td>
<td>10</td>
<td>15</td>
<td>0.54 (0.29-1.00) 0.053</td>
</tr>
</tbody>
</table>

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1505
and clear cell subtypes. Serous and mucinous subtypes might arise from ovarian epithelium responsive to stimulation of FSH while the endometrioid and clear cell subtypes might develop from ectopic endometrium in endometriosis. These findings need to be confirmed in a much larger series of cases. Such knowledge may be important in selecting patients for ovulation induction therapy. The functional aspect of these SNPs in ovarian cancer development will be investigated in the future, especially to elaborate the effects of these SNPs on the binding affinity to the FSH hormone.

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Conflict of Interest Statement: None declared.

References


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