Introduction

Endometriosis is a chronic, benign, estrogen-dependent disorder in women of reproductive age. It is characterized by the presence of ectopic endometrial tissue outside of the normal location (endometrial cavity) - mainly in the pelvic peritoneum, the ovaries, and the myometrium [1]. Clinical features of endometriosis include dysmenorrhea, deep dyspareunia, chronic pelvic pain, and infertility [2]. The development of endometriosis is regulated by enzymes and receptors that are involved in biosynthesis and metabolism of estrogens [1,3,4]. Therefore, inhibition of estradiol as the strategy of endometriosis therapy has been actively studied [5,6].

Estradiol, the most active form of estrogens, is produced either from testosterone catalyzed by aromatase (CYP19) or from estrone catalyzed by 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1) (Fig. 1) [7]. In the human endometrium, inactivation of estradiol to estrone is induced by 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) [8]. The enzyme 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3) converts androstenedione to testosterone, a precursor of estradiol [9]. In addition, two cytochrome P450 enzymes, cytochrome P450IA1 (CYP1A1) and cytochrome P450IB1 (CYP1B1), are responsible for the hydroxylation of 2-OH and 4-OH catechol estrogens which in turn induce DNA damage and mediate estrogen-induced carcinogenesis [10,11]. Catechol-O-methyltransferase (COMT) inactivates 2-OH and 4-OH catechol estrogens by catalyzing the transfer of a methyl group from S-adenosyl-methionine to a hydroxyl group on a catechol nucleus [12].

The risk of endometriosis is related to genetic factors [13,14]. Various single nucleotide polymorphisms (SNPs) have been associated with different susceptibilities to endometriosis [7,15–
Our previous study has also shown that non-synonymous SNPs of FSH receptor gene [GG genotype (680Ser/Ser) and GA genotype (680Ser/Asn)] are related to a significantly lower risk of endometriosis [19]. HSD17B1 was also found to have profound species-related polymorphisms that resulted in different efficacies of steroid conversion during drug screening [20]. Collectively, endometriosis is thought to be determined by genetic background, and individual genetic variations that may interfere with local production and circulating levels of estrogen are likely to play roles in the development of endometriosis [21].

Matrix-assisted laser desorption-ionization (MALDI) was developed for ionizing and mass-analyzing large biomolecules [22]. In addition, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used for analysis of mini-sequencing products and SNP genotyping with advantages of time-saving, absolute results, and feasible automation for high throughput analysis [23–25].

Non-synonymous SNPs (nsSNPs) [26] may account for half of the known genetic variations linked to human inherited diseases [27]. Through changing amino acids of substrates or key flanking amino acids, nsSNPs may affect protein post-translational modifications (PTMs) such as phosphorylation and glycosylation. In the database dbPTM [28,29], information of protein modifications and numerous amino acid variants associated with PTMs has been comprehensively compiled. dbPTM provides useful predictions about how non-synonymous SNPs may influence post-translational modifications of proteins. Additional computational methods, such as those used in KinasePhos [23–25,30], can be used to study how non-synonymous SNPs influence protein phosphorylation by identifying kinase-specific protein phosphorylation sites in proteins.

In this study of more than 600 Chinese women, we have used MALDI-TOF MS to systematically genotype a total of 34 nsSNPs in genes that are involved in estrogen biosynthesis and metabolism. In addition to the characterization of 22 nsSNPs that exhibit a uniformed homozygosity unique to this Chinese population, we have identified the prevalence of genotypes of nsSNPs in FSHR at positions of 307 and 680. We also identified an association between mutant genotypes in FSHR, HSD17B3 and CYP19 and decreased risks of endometriosis. Results of bioinformatics analyses suggest the functional roles of such genetic variations in the related risk of endometriosis.

Materials and Methods

Subjects

Three hundred patients of ovarian endometrioma undergoing laparotomy or laparoscopy with further pathological confirmation at Chang Gung Memorial Hospital were included as previously described [19]. The scoring system revised by the American Society for Reproductive Medicine in 1997 was used to classify the stages of endometriosis. Another 337 postmenopausal women without any history of infertility, dysmenorrhea, endometriosis/adnepoysis, and surgeries for obstetrical/gynecological diseases were recruited to be healthy controls. All of the patients in the study were Taiwanese Chinese. Informed consents were obtained from all participants. The study was approved by the local Institutional Review Board (IRB #94–975B). Blood samples (3 ml) were collected in heparinized tubes from all of patients in both groups [19]. Serum specimens were collected from another study, which was also approved by the local Institutional Review Board (IRB #98–1995A3).

Non-synonymous Single Nucleotide Polymorphisms of Estrogen Synthesis and Metabolism-related Genes

Nine genes that regulate the biosynthesis and metabolism of estrogen (Fig. 1) were studied. They were CYP19 (aromatase), CYP1A1 (cytochrome P450 1A1), CYP1B1 (cytochrome P450 1B1), HSD17B1 (17β-hydroxysteroid dehydrogenase I), HSD17B2 (17β-hydroxysteroid dehydrogenase II), HSD17B3 (17β-hydroxysteroid dehydrogenase III), ERα (estrogen receptor α), FSHR (FSH receptor), and COMT (catechol-O-methyl transferase). A total of 34 nsSNPs, listed in Table S1, were chosen according to the database of National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/SNP/).

Extraction of DNA

Genomic DNA from leukocyte in peripheral blood was extracted using a commercial kit, QiAmp DNA blood Midi Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s recommendation.

SNP Analysis by Matrix-assisted Laser Desorption-ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)

The MALDI-TOF MS SNP genotyping procedures were formatted for 96-wells [19,31,32]. Primers for the PCR and miniprimer extension reaction are shown in Table S1. Genomic regions spanning the respective SNP were amplified from each sample DNA. PCR amplification was performed in a final volume of 10 μl containing 5 ng of genomic DNA, 1X PCR buffer, 100 μM each of dTTP, dATP, dCTP, and dGTP, 1 μM each of primers, and 1U Taq DNA polymerase followed by 3 min denaturation at 95°C and 40 cycles of denaturation at 95°C for 30 sec, annealing at Tm of each primer set for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 2 min. All thermal cycles were run on a thermocycler (MJ Research, Watertown, MA, USA). Amplified double stranded DNA was isolated using GenoPure DS purification kit (Bruker Daltonics, Bremen, Germany) with automated liquid handlers, MAP-II8 and PureDisk (Bruker Daltonics, Bremen, Germany).
Allele-specific primer extension reaction was catalyzed by Thermo Sequenase (Amersham Pharmacia, Amersham, UK) at 94°C for 8 sec, 52°C for 8 sec, and 72°C for 8 sec, for 50 cycles. Primer extension products were treated with GenoPure Oligo purification kit (Bruker Daltonics, Bremen, Germany) to remove salts in the reaction buffer.

The matrix 3-hydroxypicolinic acid (3-HPA) (Fluka, Buchs, Switzerland) was used in a concentration of 10 mg/ml containing 1 mg/ml di-ammonium hydrogenate citrate. Half μl of matrix was first spotted with the MAP II/8 robotic system on the AnchorChip and allowed to dry, and then 0.5 μl of primer extension product was loaded to the dried matrix. Finally, 0.5 μl of 75% acetonitrile was added to the sample followed by MALDI-TOF MS (Autoflex, Bruker Daltonics, Germany) analysis.

Measurement of Serum Estradiol (E2)

Serum estradiol levels were assayed with the electrochemiluminescence immunoassay "ECLIA" on Elecsys and cobas e Immunoassay analyzer (Roche, Basel, Switzerland) in a College of American Pathologist (CAP)-certified laboratory. The estradiol assay sensitivity was 3 pg/ml, and the intra- and inter-assay coefficients of variation were 1.5% and 6.2%, respectively.

Statistical Analysis

The Chi-square ($\chi^2$) test was used to compare genotype distributions between patients with endometriosis and controls. Hardy-Weinberg equilibrium was examined using a goodness-of-fit test with one degree of freedom in order to compare the observed genotype frequencies with the expected genotype frequencies among study subjects. The dominant effect was analyzed by comparing one homozygous genotype (MM) to the other homozygous (NN) and the other homozygous (MN). The statistical modeling of univariate logistic regression was used to calculate the odds ratio (OR) of genetic effects. Statistical analyses were conducted by the Statistical Analysis System (SAS) software (version 8.1 for univariate logistic regression was used to calculate the odds ratio and the other homozygous (NN). The statistical modeling of univariate logistic regression was used to calculate the odds ratio (OR) of genetic effects. Statistical analyses were conducted by the Statistical Analysis System (SAS) software (version 0.1 for windows; SAS Institute Inc., Cary, NC). A P value of <0.05 was considered statistically significant. Results are presented as OR and the 95% confidence interval (CI).

Functional Network Analysis of Key Proteins

Procedures of networks analysis were similar to what we previously reported [33–35]. Briefly, we used the “analyze networks” algorithm in MetaCore (GeneGo, St. Joseph, MI) to build the networks that consisted of FSHR, HSD17B3 and CYP19. MetaCore includes a curated database of human protein interactions and metabolism; thus, it is useful for analyzing a cluster of genes in the context of regulatory networks and signaling pathways [36]. For the network analysis of a group of genes, MetaCore can be used to calculate the statistical significance $p$ value based on the probability of assembly from a random set of nodes (genes) of the same size as the input list [36].

Computational Analysis of Non-synonymous SNPs for their Effects on Post-translational Modification

To examine how nsSNPs affect post-translational protein modifications leading to changes in estrogen synthesis and metabolism, we looked into multiple databases that were previously reported [28]. Protein annotations were obtained from UniProt [37], which is a repository of protein properties. Information on protein glycosylation and phosphorylation associated with non-synonymous SNPs were obtained from dbPTM [29]. To identify the protein phosphorylation sites associated with non-synonymous SNPs, we adopted a well tested method - KinasePhos [23–25,30] to identify kinase-specific phosphorylation sites against amino acids changed by non-synonymous SNPs. KinasePhos, which is a computational tool developed by our group based on Hidden Markov models, can accurately identify kinase-specific protein phosphorylation sites [23]. Taking the polymorphism of FSH receptor gene (FSHR; Asn680Ser caused by A→G) as an example, 680Tm but not 680A in FSHR might be a potential phosphorylation site. Thus, the phosphorylation status of amino acids 680 may be affected by the polymorphism. In this study, all of the significant disease-associated non-synonymous SNPs were computationally analyzed for their influences on protein phosphorylation and glycosylation.

Results

Demographics of Studied Groups

Ages of patients with endometriosis ranged from 21 to 42 years (mean age, 34.3 years) whereas the ages of normal controls ranged from 45 to 61 years (mean age, 52.2 years). Body mass indices (kg/m^2) of both groups were similar (ranged from 16.7 to 30.9 for patients with endometriosis and from 17.2 to 31.6 for normal controls) (Table 1). Stages of endometriosis were classified according to the revised scoring system proposed by American Society for Reproductive Medicine. Most patients with endometriosis recruited in the present study were in advanced stages (III and IV, 80.4%) (Table 1). Women with endometriosis had a significant ($P<0.00001$) lower parity than healthy controls. Additionally, endometriosis patients who were older than 37 years when they first sought medical treatment had a significantly higher parity than those who first sought medical treatment younger than 37 years ($P<0.00001$) (Table 1).

Age differences between endometriosis group (34.3±6.9) and controls (52.2±4.2) might partly account for the parity difference. In addition, this parity difference might reflect a higher incidence of infertility in endometriosis patients. Moreover, our data also suggested that endometriosis-related infertility was correlated with the age at diagnosis, as older endometriosis group (>37 years old) who sought medical treatment for the first time had a significantly higher parity than younger ones ($P<0.00001$) (Table 1). These findings implied that patients with earlier onset of endometriosis suffered more from infertility.

Serum Estradiol (E2) Levels in Patients with Surgically Confirmed Endometriosis Before and After Operation

In an independent retrospective study, serum estradiol levels in a cohort of 100 patients before and after operation were measured. Sera obtained from 100 age-matched women were measured as controls. There was not significant difference in serum levels of estradiol in these three groups (Table S1).

Chinese Preferential Homozygosity of Non-synonymous SNPs in Estrogen Synthesis and Metabolism-related Genes

Among 34 nsSNPs genotyped in this study (Table S2), 22 were found to be homozygous in more than 600 Taiwanese women. These 22 SNPs were CYP19 (rs2304462, GG homozygous; rs1803154, AA homozygous), CYP1A1 (rs1799814, CC homozygous; rs2229150, CC homozygous; rs2278970, GG homozygous; rs2856833, CC homozygous; rs4987133, GG homozygous; rs515159, AA homozygous; rs8191136, GG homozygous), HSD17B2 (rs1911136, GG homozygous), HSD17B3 (rs1800440, AA homozygous; rs4398235, TT homozygous; rs4986887, GG homozygous; rs4986888, CC homozygous).
Table 1. Characterization of the studied population.

| Cases of endometriosis | Normal controls | p* |
|------------------------|-----------------|----|
| (n = 300)              | (n = 337)       |    |
| Age                    | 34.3 ± 6.9      | 52.2 ± 4.2 | <0.00001 |
| Body mass index (kg/m²) | 22.0 ± 3.4      | 23.6 ± 3.1 | NS        |
| Parity                 | 1.1 ± 1.1       | 2.6 ± 1.1 | <0.00001 |
| >37 years in endometriosis (n = 124) | 1.6 ± 1.1  | 2.6 ± 1.1 | <0.00001 |
| <37 years in endometriosis (n = 176) | 0.7 ± 0.7 | 2.6 ± 1.1 | NS        |

Stage of endometriosis

| III                     | 55.0% (n = 165) |    |
| IV                      | 25.4% (n = 76)  |    |

*Student-t test.
NS: not significant.
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(rs2066480, GG homozygous), ERα (rs9340773, GG homozygous; rs17847065, CC homozygous; rs17847076, AA homozygous), FSHR (rs6167, CC homozygous; rs1126714, CC homozygous), and COMT (rs6270, GG homozygous; rs6267, GG homozygous; rs5031015, GG homozygous; rs4986871, CC homozygous).

Mutant SNPs of HSD17B3 and CYP19 Showed a Lower Risk in Endometriosis Patients Younger than 37 Years

In all endometriosis patients (n = 300), a univariate analysis for the Gly289Ser (G/A) polymorphism of 17β-hydroxysteroid dehydrogenase III (HSD17B3) revealed that only homozygous mutant SNP (genotype AG, 289Ser/Gly) of HSD17B3 showed a significantly decreased risk of endometriosis (P = 0.03; OR = 0.7) as compared to the controls (n = 337). In contrast, in endometriosis patients younger than 37 years (n = 176), combined homozygous and heterozygous mutant SNP (genotype AA+AG, 289Ser/289Ser+289Ser/Gly) of HSD17B3 showed a significantly lower risk of endometriosis (P = 0.003; OR = 0.7) as compared to the controls (n = 337) (Table 2). In contrast, in younger endometriosis patients (n = 176), homozygous mutant SNP of CYP19 (genotype TT, 264Cys/Cys) showed a significantly lower risk of endometriosis (P = 0.003; OR = 0.71) when compared to the controls (n = 337) (Table 3). This result was not shown when both younger and older groups were combined (n = 300) (Table 2).

A SNP of FSHR at Position 680 in Combination with SNPs in HSD17B3, CYP19 or FSHR at Position 307 showed Decreased Risks of Endometriosis

In regard to the Asn680Ser (A/G) polymorphism of FSHR and the Gly289Ser (G/A) polymorphism of HSD17B3, univariate analyses on all endometriosis women (n = 300) revealed that a combination of homozygous/heterozygous mutants of FSHR (genotype GG+GA, 680Ser/Ser+680Ser/Asn) and homozygous/heterozygous mutants of HSD17B3 (genotype AA+AG, 289Cys/Cys+289Cys/Gly) was associated with significantly decreased risk of endometriosis (P = 0.00002; OR = 0.46) as compared to the combination of homozgyous wild types of FSHR and HSD17B3 (Table 4).

Similarly, a significantly decreased risk for endometriosis was found in women who had at least a mutant allele of FSHR (genotype GG+GA, 680Ser/Ser+680Ser/Asn) and CYP19 (genotype TT+TC, 264Cys/Cys+264Cys/Asn) (P = 0.01; OR = 0.66) (Table 4). Furthermore, a significantly decreased risk for endometriosis was also observed in women who had at least a mutant allele in FSHR at position 680 (genotype GG+GA, 680Ser/Ser+680Ser/Asn) and position 307 (genotype GG+GA, 307Ala/Ala+307Ala/Thr) (P = 0.01; OR = 0.66) (Table 4).

Two Non-synonymous SNPs of FSHR at Positions 307 (rs6165) and 680 (rs6166) in Chinese Women were not in the Same Haplotype

In all women studied, frequencies of genotype with combined homozygous SNPs in FSHR at positions 307 (rs6165) and 680 (rs6166) (both wild-type alleles, 307Thr/Thr680Ser/Ser) were 39.4% and 9.6% respectively. In contrast, 39.8% of women studied possessed genotypes of combined heterozygous SNPs in FSHR (307Thr/Thr680Ser/Asn) (Table 5). Furthermore, at least a heterozygous SNP in FSHR at positions 307 (rs6165) and 680 (rs6166) was found in 51.0% of Taiwanese Chinese women studied. These results indicated that, even though these 2 SNPs reside in the exons of the same gene, they are not in the same haplotype.

Functional Networks Among FHSR, HSD17B3 and CYP19

Using MetaCore algorithm for networks analysis, we found that FSHR, HSD17B3 and CYP19 interacted in the network of pathways with a P value of 1.5 x 10^-105 (Fig. 2), indicating that the probability of assembly from random sets of nodes (genes) was very low [30]. The pathways of FHSR and those of HSD17B3 and CYP19 intersected at the androgen receptor (AR).

Modification of Protein Glycosylation and Phosphorylation by Non-synonymous SNPs

Figure 3 depicts the membrane topology of FSHR, an O-linked glycosylated amino acids at 307Thr, and a phosphorylated amino acids at 680Ser. Based on the statistics of UniProt membrane proteins, 212 of 216 O-linked glycosylation sites occur.
### Table 2. Genotype frequency and overall association with estrogen synthesis and metabolism-related genes for women with endometriosis (n = 300) and controls (n = 337).

| Genes | SNP ID | Genotypes | Cases (%) | Controls (%) | OR   | 95% CI | P * |
|-------|--------|------------|-----------|--------------|------|--------|-----|
| CYP1A1 | rs1048943 | A: Ile Ile462Val (A→G) | AA 167 (55.7) | 182 (54.0) | 1.00 | Ref. - |    |
|       |        | G: Val     | AG 110 (36.7) | 134 (39.8) | 0.90 | 0.64–1.26 | 0.51 |
|       |        | GG 23 (7.7) | 21 (6.2) | 0.99 | 0.77–1.29 | 0.97 |
|       |        | AG+GG 133 (44.3) | 155 (46.0) | 0.94 | 0.67–1.29 | 0.67 |
| CYP1A1 | rs4646422 | G: Gly Gly45Asp (G→A) | GG 214 (71.3) | 251 (74.5) | 1.00 | Ref. - |    |
|       |        | A: Asp     | AG 81 (27.0) | 79 (23.4) | 1.18 | 0.78–1.92 | 0.39 |
|       |        | AA 5 (1.7) | 7 (2.1) | 1.09 | 0.69–1.82 | 0.68 |
| CYP1B1 | rs10012 | C: Arg Arg48Gly (C→T) | CC 202 (67.3) | 238 (70.6) | 1.00 | Ref. - |    |
|       |        | G: Val     | CG 51 (17.0) | 63 (19.1) | 0.88 | 0.57–1.35 | 0.54 |
|       |        | GG 0 (0) | 3 (0.9) | 0.82 | 0.55–1.21 | 0.29 |
|       |        | CG+GG 51 (17.0) | 66 (20.0) | 0.84 | 0.55–1.28 | 0.40 |
| HSD17B3 | rs2066479 | G: Gly Gly289Ser (G→A) | GG 186 (62.0) | 186 (55.2) | 1.00 | Ref. - |    |
|       |        | A: Ser     | AG 53 (17.7) | 68 (20.2) | 0.97 | 0.77–1.22 | 0.77 |
|       |        | AA 20 (6.7) | 16 (4.8) | 0.87 | 0.67–1.14 | 0.31 |
| FSHR | rs6165 | A: Thr Thr307Ala (A→G) | AA 140 (46.7) | 156 (46.3) | 1.00 | Ref. - |    |
|       |        | G: Ala     | AG 122 (40.7) | 135 (40.0) | 0.73 | 0.52–1.04 | 0.07 |
|       |        | GG 38 (12.7) | 46 (13.7) | 0.85 | 0.67–1.08 | 0.17 |
| FSHR | rs6166 | A: Asn Asn680Ser (A→G) | AA 148 (49.3) | 126 (37.4) | 1.00 | Ref. - |    |
|       |        | G: Ser     | AG 121 (40.3) | 173 (51.0) | 0.60 | 0.42–0.84 | 0.002 |
|       |        | GG 31 (10.3) | 38 (11.3) | 0.75 | 0.59–0.95 | 0.02 |
|       |        | AG+GG 152 (50.7) | 211 (62.6) | 0.61 | 0.44–0.86 | 0.002 |
**Table 2. Cont.**

| Genes | SNP ID. No. | Genotypes | Cases (%) | Controls (%) | OR | 95% CI | P * |
|-------|-------------|-----------|-----------|--------------|----|--------|----|
| COMT  | rs4680      | G: Val    | 171 (57.0)| 194 (57.6)   | 1.00| Ref.   | –  |
|       |             | Val158Met (G→A) |          |              |    |        |    |
|       |             | A: Met    | 111 (37.0)| 116 (34.4)   | 1.09| 0.77–1.53| 0.63|
|       |             | AA        | 18 (6.0)  | 27 (8.0)     | 0.96| 0.74–1.25| 0.77|
|       |             | AG+AA     | 129 (43.0)| 143 (42.4)   | 1.02| 0.74–1.42| 0.89|

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

**Discussion**

It is important to choose appropriate controls in association studies, because selection bias adversely affects results [40]. Previously, control groups have been selected from female newborns from the same ethnic group as the population control [41] or the women drawn from the same clinic population who were free of endometriosis [40]. Women with laparoscopic confirmation of free of endometriosis seem to be rational controls; however, such groups may develop endometriosis later in the life. Although laparoscopy remains the gold standard approach to confirm endometriosis [42], this invasive procedure is never done without medical indications, such as chronic pelvic pain and adnexal masses. Such potential co-morbidities may exclude these women from healthy controls. Therefore, the non-endometriosis controls in this study were chosen from postmenopausal women aged 45 years or older (range: 45–61 years; mean: 52.2 years), who had no history of infertility and dysmenorrhea, no previous history for obstetrical or gynecological diseases. Accordingly, we did not adjust the odds ratios by age between endometriosis cases and controls in this study.

Several SNPs of the estrogen synthesis- and metabolism-related genes, such as CYP19 [7,15,18], CYP1A1 and COMT [43], and CYP1B1 [44], have been found to be associated with increased risk of endometriosis. However, among the 35 NCBI-listed non-synonymous SNPs [including a SNP (rs6166) that was published at extracellular regions in 49 membrane proteins [37]. Thus, FSHR 307Thr (wild type), which is located extracellularly, was identified as an O-linked glycosylation site by dbPTM (See http://dbptm.mbc.nctu.edu.tw/search_result.php?search_type = db_id&swiss_id = FSHR_HUMAN, and http://www.uniprot.org/uniprot/P23945). The flanking sequence of 307Thr has a similar composition of amino acids to those experimentally verified O-linked glycosylated threonine. A sequence logo [39] is presented to illustrate the amino acids composition of O-linked glycosylation substrate (Fig. 3). Using KinasePhos [23,25], FSHR 680Ser (mutant) was identified as a phosphorylation site that may be catalyzed by protein kinase B (PKB), protein kinase A (PKA), or ribosomal protein S6 kinase (RSK) (See http://ca.expasy.org/cgi-bin/variant_pages/get-sprot-variant.pl?VAR_013903). According to KinasePhos, an arginine (R) at the -3 position of 680Ser of FSHR variant is similar to the motif of PKB phosphorylated serine, which requires an arginine (R) at -3 position. Likewise, HSD17B3 289Ser (mutant) was also identified to be a phosphorylation site that may be catalyzed by PKB or RSK1 (See http://dbptm.mbc.nctu.edu.tw/search_result.php?search_type = db_id&swiss_id = HSD17B3_HUMAN, http://www.uniprot.org/uniprot/P37058, and http://ca.expasy.org/cgi-bin/variant_pages/get-sprot-variant.pl?VAR_014871).

**Table 3.** Genotype frequency of single nucleotide polymorphisms and overall association with estrogen synthesis and metabolism-related genes for women with endometriosis aged younger than 37 years (n = 176) and controls (n = 337).

| Genes | SNP ID. No. | Genotypes | Cases (%) | Controls (%) | OR | 95% CI | P * |
|-------|-------------|-----------|-----------|--------------|----|--------|----|
| HSD17B3 | rs2066479  | G: Gly    | 119 (68.6)| 186 (53.2)   | 1.00| Ref.   | –  |
|        |            | Gly289Ser (G→A)|          |              |    |        |    |
|        |            | A: Ser    | 49 (27.8)| 135 (40.1)   | 0.57| 0.39–0.86| 0.005|
|        |            | AG        | 8 (4.6)  | 16 (4.8)     | 0.69| 0.49–0.96| 0.02 |
|        |            | AA        | 57 (32.4)| 151 (44.8)   | 0.59| 0.39–0.88| 0.007|
|        |            | AG+AA     | 12 (7.0) | 35 (10.4)    | 0.40| 0.24–0.65| 0.001|
| CYP19  | rs700519   | C: Arg    | 132 (75.0)| 242 (71.8)   | 1.00| Ref.   | –  |
|        |            | Arg264Cys (C→T)|         |              |    |        |    |
|        |            | T: Cys    | 24 (13.6)| 26 (8.3)     | 1.57| 0.83–2.94| 0.13|
|        |            | TT        | 20 (11.4)| 67 (19.9)    | 0.71| 0.50–0.98| 0.03 |
|        |            | CT+TT     | 44 (25.0)| 95 (28.2)    | 0.85| 0.55–1.31| 0.44|

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

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At extracellular regions in 49 membrane proteins [37]. Thus, FSHR 307Thr (wild type), which is located extracellularly, was identified as an O-linked glycosylation site by dbPTM (See http://dbptm.mbc.nctu.edu.tw/search_result.php?search_type = db_id&swiss_id = FSHR_HUMAN, and http://www.uniprot.org/uniprot/P23945). The flanking sequence of 307Thr has a similar composition of amino acids to those experimentally verified O-linked glycosylated threonine. A sequence logo [39] is presented to illustrate the amino acids composition of O-linked glycosylation substrate (Fig. 3). Using KinasePhos [23,25], FSHR 680Ser (mutant) was identified as a phosphorylation site that may be catalyzed by protein kinase B (PKB), protein kinase A (PKA), or ribosomal protein S6 kinase (RSK) (See http://ca.expasy.org/cgi-bin/variant_pages/get-sprot-variant.pl?VAR_013903 and http://ca.expasy.org/cgi-bin/variant_pages/get-sprot-variant.pl?VAR_013905). According to KinasePhos, an arginine (R) at the -3 position of 680Ser of FSHR (Fig. 3) is similar to the motif of PKB phosphorylated serine, which requires an arginine (R) at -3 position. Likewise, HSD17B3 289Ser (mutant) was also identified to be a phosphorylation site that can be catalyzed by PKB or RSK1 (See http://dbptm.mbc.nctu.edu.tw/search_result.php?search_type = db_id&swiss_id = HSD17B3_HUMAN, http://www.uniprot.org/uniprot/P37058, and http://ca.expasy.org/cgi-bin/variant_pages/get-sprot-variant.pl?VAR_014871).

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previously in [19]), twenty two in eight genes (significantly lower parity than 37 years when they first sought medical treatment had a

Table 4. Combined genotypes of two single nucleotide polymorphisms (mutant, homozygous) in women with endometriosis (n = 300) and controls (n = 337).

| Combined genotypes of two SNPs | Cases (%) | Controls (%) | OR** | 95% CI** | P* |
|-------------------------------|-----------|--------------|------|----------|----|
| FSHR (rs6166, AA)+HSD17B3 (rs2066479, GG) | 99 (33.0) | 62 (18.4) | 1.00 | Ref. |
| FSHR (rs6166, GG)+HSD17B3 (rs2066479, AA+AG) | 201 (67.0) | 275 (81.6) | 0.46 | 0.31–0.67 | 0.00002 |
| FSHR (rs6166, AA)+FSHR (rs6165, AA) | 109 (36.3) | 92 (27.3) | 1.00 | Ref. |
| FSHR (rs6166, GG)+FSHR (rs6165, GG+GA) | 191 (63.7) | 245 (72.7) | 0.66 | 0.46–0.93 | 0.01 |
| FSHR (rs6166, AA)+FSHR (rs6165, AA) | 134 (44.7) | 117 (34.7) | 1.00 | Ref. |
| FSHR (rs6166, GG)+FSHR (rs6165, GG+GA) | 166 (55.3) | 220 (65.3) | 0.66 | 0.47–0.92 | 0.01 |
| FSHR (rs6166, AA)+COMT (rs46860, GG) | 90 (30.0) | 72 (21.4) | 1.00 | Ref. |
| FSHR (rs6166, GG)+COMT (rs46860, AA+AG) | 210 (70.0) | 265 (78.6) | 0.63 | 0.43–0.92 | 0.01 |

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 5. Non-synonymous SNPs of FSHR at positions 307 (rs6165) and 680 (rs6166) in Chinese women.

| FSHR (rs6165) | FSHR (rs6166) | Cases of endometriosis (n = 300) | Controls (n = 337) | Cases+Controls (n = 637) | P% |
|---------------|---------------|---------------------------------|-------------------|-------------------------|----|
| 307Thr/Thr    | 608Ser/Asn    | 134                              | 117               | 251                     | 39.4 |
| 307As/Thr     | 608Ser/Asn    | 111                              | 136               | 247                     | 38.8 |
| 307As/As      | 608Ser/Asn    | 28                               | 33                | 61                      | 9.6  |
| 307Thr/Thr    | 608Ser/Asn    | 5                                | 33                | 38                      | 6.0  |
| 307As/As      | 608Ser/Asn    | 9                                | 10                | 15                      | 2.3  |
| 307Thr/Thr    | 608Ser/Asn    | 5                                | 3                 | 12                      | 1.9  |
| 307As/As      | 608Ser/Asn    | 2                                | 1                 | 3                      | 0.5  |
| 307Thr/Thr    | 608Ser/Asn    | 1                                | 1                 | 2                      | 0.3  |

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.
wild-type homozygous polymorphism of FSHR gene (680\textsuperscript{Asn/Asn}) induces higher aromatase activity than mutant FSHR gene, resulting in production of more estrogens and stimulating proliferation of endometriotic tissues [19]. Collectively, the GG homozygous genotype of polymorphism of HSD17B3 (289\textsuperscript{Gly/Gly}) (wild type) may play a crucial role in the development of endometriosis in the presence of AA homozygous genotype of polymorphism of FSHR (680\textsuperscript{Asn/Asn}) (wild type).

In the present study, the frequencies of completely combined homozygous SNPs in FSHR at positions 307 and 680 (307\textsuperscript{Thr/Thr680\textsuperscript{Asn/Asn}}, wild-type homozygous, and 307\textsuperscript{Ala/Ala680\textsuperscript{Ser/Ser}}, mutant homozygous) were 39.4\% and 9.6\% while the frequency of at least a heterozygous SNP in FSHR at positions 307 and 680 was 51.0\% (Table 5). These findings were similar to those in a Japanese population reported previously [49,50]. Clinically, the 680\textsuperscript{Ser} allele was associated with lower sensitivity to FSH during ovulation induction [51]. Similarly, a higher dose of exogenous FSH is required to achieve ovulation induction in women with FSHR genotype 307\textsuperscript{Thr/Thr680\textsuperscript{Asn/Asn}} (mutant homozygous) [49,50]. In addition, FSHR with alleles 307\textsuperscript{Thr/Thr680\textsuperscript{Asn/Asn}} (wild-type homozygous) possesses higher bioactivity of intracellular transduction and aromatase after binding to FSH, since women with genotype 307\textsuperscript{Thr/Thr680\textsuperscript{Asn/Asn}} are more likely to develop severe ovarian hyperstimulation syndrome (OHSS) during ovulation induction with FSH [52]. In summary, FSHR with completely wild-type homozygous SNPs at positions 307 and 680 (307\textsuperscript{Thr/Thr680\textsuperscript{Asn/Asn}}) had higher sensitivity to FSH and an increased risk of endometriosis, whereas FSHR possessing at least an allele of mutant SNP at positions 307 and 680 lower risk of endometriosis.

Non-synonymous SNP in CYP19 gene (Arg264Cys, C\textsuperscript{\rightarrow}T) alone was not correlated with the change in risk of endometriosis (Table 2). However, in the presence of mutant SNP in FSHR gene (680\textsuperscript{Ser/Ser680\textsuperscript{Ser/Asn}}), mutant SNPs in CYP19 gene demonstrated a significantly decreased risk of endometriosis (Table 4). The binding of FSH to FSHR activates aromatase (CYP19), which in turn induces estrogen production [7]. In the presence of mutant SNP in FSHR gene (680\textsuperscript{Ser/Ser680\textsuperscript{Ser/Asn}}), mutant SNPs in CYP19 gene demonstrated a significantly decreased risk of endometriosis (Table 4), indicating that FSHR and CYP19 had synergistic effects on the production of estrogen. On the contrary, the COMT polymorphism (rs4680) was not associated with the risk of endometriosis, which is in agreement with a recent report of a Brazilian population [53].
Revealed by network analysis using MetaCore (Fig. 2), the interaction among FSHR, CYP19 and HSD17B3 at the androgen receptor (AR) may have a clinical importance. Active androgens and AR are shown in endometriotic lesion of women with stage III or IV disease, suggesting that endometriotic tissues respond to androgens [54]. As shown in Fig. 2, FSHR activation recruits beta-arrestin2 for its desensitization and internalization [55]. Beta-arrestin2 inhibits AR directly and acts as a corepressor of AR by serving as a scaffold for Mdm2, leading to the ubiquitination and degradation of AR [56]. On the other hand, HSD17B3 may stimulate AR through the regulation of cytoplasmic testosterone metabolism [57].

Our results also support the role of AR in the pathophysiology and therapeutics of endometriosis. First, danazol, an androgen analog used for treatment of endometriosis, directly binds to the AR of endometriotic tissue [58] and decreases the expression of Bcl-2 (a suppressor for apoptosis) [59], resulting in the cell death of endometriotic tissue. Second, an animal study has shown that danazol in vivo reduces AR, estrogen receptors, and progesterone receptors of the endometrium [60]. Third, an in vitro study has demonstrated that the toxic effects on the endometrial stromal cells by danazol, such as destruction of cell organelles and cytoskeleton, were mainly mediated by androgen receptors [61].

Bioinformatics using databases in this investigation provided useful predictions for conformational changes of proteins affected by nsSNPs. Our results suggest that glycosylated 307Thr of FSHR may mediate extracellular recognition events, which may be important in the development of endometriosis. In addition, the significant associations between the conversion of 680Asn to 680Ser, resulted from A to G of rs6166, and endometriosis (Tables 2 to 4) suggest that phosphorylation of the cytoplasmic residue 680Ser may be important for normal signaling pathways against the development of endometriosis. Furthermore, conversion of 289Gly to 289Ser (G to A of rs2066479) of HSD17B3 is associated with a decreased risk of endometriosis in younger women (Table 3). Although Ser/Gly of residue 289 of HSD17B3 was proposed to be a neutral polymorphism [62], we found that the phosphorylation of 289Ser in HSD17B3 may decrease the risk of endometriosis.

Polymorphisms in promoter regions of genes have been shown to affect the levels of gene expression. For instance, promoter polymorphism of interleukin-10 gene (rs180087) was recently shown to be associated with the risk of endometriosis [63]. Polymorphisms in regulatory elements of genes may be localized at the site for methylation, which may change the susceptibility of gene silencing. On the other hand, non-synonymous SNPs in
exons change the conformation of proteins, likely affecting protein functions, especially in an enzyme [27].

Inhibition of estrogen itself or estrogen-related steroid conversion pathways [Fig. 1] has been actively studied for the development of targeted therapy for endometriosis [3,6]. It is conceivable that the designer’s drugs aiming at endometriosis-specific structural changes of key proteins may exert the greatest efficacy against disease but spare undesirable effects against enzymes with normal structures. Our results did not identify endometriosis-specific amino acid changes in HSD17B1 (Table 2) but detected endometriosis preferential structural changes of HSD17B3 (Table 3), CYP19 (Table 3), and FSHR (Table 4, and Fig. 3). These findings may help us design disease-specific, targeted therapy. For instance, the extracellular domains of FSHR are theoretically targetable regions by drugs that are delivered by circulating blood. The higher prevalence of 307Thr of FSHR (Fig. 3) makes it a highly rational target for drug development in endometriosis therapy. Similarly, drugs that aim at the domain of HSD17B3 containing 286Glu may be more beneficial for the treatment of severe endometriosis that frequently occurs in young women (Table 3).

In conclusion, our results identified that 4 nsSNPs (rs6165, rs6166, rs2066479, rs700519) in estrogen synthesis and metabolism-related genes may decrease the risk of endometriosis. Because these 4 nsSNPs reside in 3 genes related to estrogen synthesis (HSD17B3, FSHR and CYP19) (Fig. 1), endogenous production of more estrogens, instead of slowing the degradation of estrogens and their metabolites, may be more strongly associated with the risk of endometriosis. Identification of the endometriosis-preferential nsSNPs and the conformational changes in those proteins may pave the way for the development of more disease-specific drugs in this devastating disease.

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Supporting Information
Table S1 Serum levels of estradiol (E2) in patients with surgically confirmed endometriosis and age-matched healthy controls.

Table S2 Genotypes and amino acid types/positions of non-synonymous single nucleotide polymorphisms (SNP) in estrogen synthesis and metabolism-related genes were obtained from National Center for Biotechnology Information (NCBI). Status of polymorphism in the population studied and primers for the first polymerase chain reaction (PCR) and extension reaction at each SNP are also shown.

Table S3 Genotypes and amino acid types/positions of non-synonymous single nucleotide polymorphisms (SNP) in estrogen synthesis and metabolism-related genes were obtained from National Center for Biotechnology Information (NCBI). Status of polymorphism in the population studied and primers for the first polymerase chain reaction (PCR) and extension reaction at each SNP are also shown.

Database Links S1 Supplementary Database Links.

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Author Contributions
Conceived and designed the experiments: HSW THW. Performed the experiments: HSW HMW PYC. Analyzed the data: HSW YSL HDH. Contributed reagents/materials/analysis tools: HSW HMW BHC CFY AC. Wrote the paper: HSW. Functional Analyses of SNP in Endometriosis

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