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Original Article

Associations between a single nucleotide polymorphism of stress-induced phosphoprotein 1 and endometriosis/adenomyosis

Chia-Lung Tsai [a, 1], Yun-Shien Lee [b, 1], Angel Chao [c, d], Chih-Feng Yen [c], Hsin-Shih Wang [c], Tzu-Hao Wang [a, c, e, *]

[a] Genomic Medicine Core Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan
[b] Department of Biotechnology, Ming-Chuan University, Taoyuan, Taiwan
[c] Department of Obstetrics and Gynecology, LinKou Medical Center, Chang Gung Memorial Hospital and Chang Gung University, Linkou, Taiwan
[d] Gynecologic Cancer Research Centre, LinKou Medical Center, Chang Gung Memorial Hospital, Linkou, Taiwan
[e] Graduate Institutes of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan

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ABSTRACT

Objective: We have recently reported that stress-induced phosphoprotein 1 (STIP1) is over-expressed in endometriosis/adenomyosis tissues. STIP1 may also be involved in immune regulation, thus we attempted to study the association between STIP1 single nucleotide polymorphisms (SNPs) and endometriosis/adenomyosis.

Materials and methods: Five STIP1 SNPs (rs7941773, rs2845597, rs4980524, rs2282490, and rs2236647) were selected for genotyping with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in 286 patients with endometriosis/adenomyosis and 288 healthy postmenopausal controls. *In vitro* studies included luciferase promoter reporter assays and western blot analysis for STIP1 and MMP9 proteins.

Results: The frequency of the G allele at rs4980524 was significantly higher in patients with endometriosis/adenomyosis than in control women. The promoter reporter with rs4980524 GG genotype significantly increased luciferase activity than that with TT genotype in endometrial cancer RL95-2 cells, and the primary endometrial stromal cells carrying rs4980524 GG genotype expressed higher protein levels of STIP1 and MMP9 than those carrying the TT one.

Conclusion: The G/G allele of STIP1 SNP rs4980524 is associated with the increased expression of STIP1 and MMP9 in endometriosis. Further validation in independent cohorts of endometriosis patients may prove its usefulness as a genetic risk maker for endometriosis/adenomyosis.

Introduction

Endometriosis is characterized by growth of ectopic endometrial tissues outside the endometrial cavity, affecting 5–10% of women of reproductive age [1]. Clinically, dysmenorrhea, deep dyspareunia, chronic pelvic pain, and infertility are commonly seen symptoms of endometriosis [2]. Despite its devastating effects on women's quality of life and reproductive potential, the etiology of endometriosis remains inconclusive. Historically, Sampson proposed that reflux menstruation might result in endometriosis [3], which is now known to be true only in the presence of impaired immunological surveillance. Meyer proposed the theory of coelomic metaplasia, predicting the pluripotency of certain groups of peritoneal mesothelium [4]. Meigs suggested that Mullerian metaplasia could be induced by abnormal hormonal influence [5]. Since these theories were proposed decades ago, thousands of studies, including immune dysfunction [1], have provided evidence that support or dispute against each theory.

Case-control association studies using genetic markers are based on the “common disease, common variant” assumption, which means that the genetic risk for a common disease can usually be attributed to a relatively small number of common genetic variants [6]. Single nucleotide polymorphisms (SNPs) are the preferred genetic markers for such studies because of their

* Corresponding author. Genomic Medicine Core Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan.
E-mail address: knoxtn@cgmh.org.tw (T.-H. Wang).
[1] C.L.T. and Y.S.L. contributed equally to this work.

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abundance; about 12 million unique human SNPs have been assigned a reference SNP number in the National Center for Biotechnology Information's SNP database [7]. The development of endometriosis is regulated by enzymes and receptors that are involved in biosynthesis and metabolism of many hormones, including estrogens [1,8]. Various SNPs have been associated with the severity of and the susceptibility to endometriosis [9,10]. Recently, we have also reported that both the GG and GA genotypes of the FSH receptor gene (Asn680Ser) are associated with a significantly lower risk of endometriosis [2].

Stress-induced phosphoprotein (STIP1, Gene ID 10963; HPRD 05454) is also known as heat shock protein (HSP)-organizing protein (HOP) [11]. STIP1 modulates the chaperone activities of HSP 90 and HSP 70 [11] and overexpresses in several malignant tissues, including melanoma [12], hepatocellular carcinoma [13], glioma [14], ovarian cancer [15] and pancreatic cancer [16]. Knockout STIP1 in mice shows embryonic lethal, activates caspase 3 and impairs cell proliferation [17]. Knocking down endogenous STIP1 with siRNA decreased the expression of HSP90 client oncoproteins (e.g., HER2, Bcr-Abl, c-MET, and v-Src) [18]. STIP1 is also involved in regulation of cell migration through modulating matrix metalloproteinase-2,9 [18,19] and RhoC GTPase [20].

Several protein interaction domains of STIP1 are identified, including three tetratricopeptide repeat (TPR) domains (1,2A, 2B) and two rich in aspartate and proline (DP) domains (1 and 2) [21]. TPR1 and 2B are essential for HSP70 binding, whereas TPR2A, TPR2B and DP2 domains involve HSP90 interaction [22–24]. STIP1 also maintains protein stability of JAK2, CDK3, survivin and AKT [24,25] through these interactions. A glioblastoma cell line has been shown to secrete STIP1 into culture medium, and recombinant STIP1 can induce proliferation of glioma cells by activating the ERK, PI3K and BMP pathways [14,26]. Treatment of ovarian cancer cells with STIP1 significantly increases ERK phosphorylation, promotes DNA synthesis, and increases Ki-67 immunoreactivity in ovarian cancer cells, extending the pro-proliferative role of STIP1 in the tumorigenesis of ovarian cancer [15].

Recently, SNPs of STIP1 were found to correlate with lung function in asthmatic subjects treated with inhaled corticosteroids, suggesting its role as a prediction marker for glucocorticosteroid responses in patients with reduced lung function [27]. Given the implication of STIP1 involvement in immune regulation, we attempted to study the association between STIP1 SNPs and endometriosis. Our results showed that STIP1 over-expression in endometriosis [19] was associated with a higher allele frequency of G in the STIP1 SNP (rs4980524) in patients with endometriosis. Further *in vitro* studies supported that the G/G allele of rs4980524 indeed stimulated STIP1 expression.

Materials and methods

Ethics statement

All procedures complied with the tenets of the Helsinki declaration and were approved by the Institutional Review Board of the Chang Gung Memorial Hospital (IRB approval #94-975B and #97-0753B).

Culture and treatment of cell lines

Human endometrial cancer cell RL95-2 was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DEME/F12 with 10% fetal bovine serum and appropriate amounts of penicillin and streptomycin.

Extraction of genomic DNA

The procedure for genomic DNA extraction and the samples subjected to STIP1 SNPs genotyping have been previously described in detail [28]. In brief, cases were women with a pathological diagnosis of endometriosis who had undergone surgical treatment. Postmenopausal women with a negative history of infertility and dysmenorrhea who were free of endometriosis and/or adenomyosis were selected as controls. All controls did not previously undergo surgery for obstetric and/or gynecologic conditions. We purposely selected postmenopausal women as controls for genotyping to reduce the likelihood of including subjects at risk of developing endometriosis later in life. All of the patients and controls included in the study were of Taiwanese descent. Genomic DNA was extracted from peripheral leukocytes using a QIAmp DNA blood Midi Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol.

DNA construction

STIP1 luciferase reporter vectors containing the rs4980524 SNP were amplified from the patients' genomic DNA based on their known genotypes. The following primers for rs4980524 were used: forward, 5′-ACGAAGCTTGCCGTGAGGCAAGATTGTGC-3′, and reverse, 5′-ACGAAGCTTCCGGTGGGATACAGCAGTTT3′. PCR conditions were as follows: initial step at 95 °C for 5 min, followed by 40 cycles of 1 min at 95 °C, 1 min at 55 °C, and 72 °C for 10 min. PCR products were digested with the restriction enzyme *Hind* III and then ligated with a *Hind* III/CIP-treated pGL4.26 vector (Promega, Madison, WI, USA). DNA sequences were confirmed with an ABI DNA autosequencer (Applied Biosystems, Foster City, CA, USA).

SNPs genotyping

Five STIP1 SNPs (rs7941773, rs2845597, rs4980524, rs2282490, and rs2236647) were selected for genotyping [27], which was conducted by MALDI-TOF MS in a 96-well format [29,30]. PCR primers and mini-primer sets are reported in Table 1. PCR amplification was performed in a final volume of 10 μL containing 5 ng of genomic DNA, 1 × PCR buffer, 100 μM of each dNTP, 1 μM of each primer, and 1U Taq DNA polymerase. PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 30 s denaturation at 95 °C, annealing for 30 s at Tm of each primer set, and extension for 30 s at 72 °C, with a final extension at 72 °C for 2 min. PCR products were purified using a GenoPure DS purification kit (Bruker Daltonics, Bremen, Germany). Allele-specific primer extension reactions were catalyzed by Thermo Sequenase™ DNA polymerase (Amersham Pharmacia, Amersham, UK) for 50 cycles of 8 s at 94 °C, 8 s at 52 °C, and 8 s at 72 °C. Primer extension products were treated with the GenoPure Oligo purification kit (Bruker Daltonics) to remove salts from the reaction buffer. Matrix 3-hydroxypicolinic acid (3-HPA) (Fluka, Buchs, Switzerland) was used at a concentration of 10 mg/mL containing 1 mg/mL diammonium hydrogen citrate. We initially spotted 0.5 μL of the matrix on the Anchor Chip using the map II/8 MALDI AutoPrep system (Bruker Daltonics). After drying, 0.5 μL of the primer extension product was loaded onto the dried matrix. Finally, we added 75% acetonitrile (0.5 μL) to the sample which was subsequently subjected to MALDI-TOF MS (Autoflex, Bruker Daltonics) analysis.

DNA transfection and luciferase reporter assay

The procedures used for DNA transfection and construction of the luciferase reporter assays have been previously described