CEOG Clinical and Experimental Obstetrics & Gynecology

-1195 A/G promoter variants of the cyclooxygenase-2 gene increases the risk of pain occurrence in endometriotic women

H. Wang, L. Sun, M. Jiang, L. Liu, G. Wang

Department of Gynecology, Dalian Hospital of Gynecology and Obstetrics, Dalian (China)

Summary

Objective: The aim of this study was to evaluate the association between -1195 A/G polymorphism in cyclooxygenase -2 (COX-2) gene and the risk of pain occurrence in women with endometriosis (EM). *Materials and Methods:* -1195 A/G polymorphism in the promoter region of COX-2 gene was analyzed in 32 EM patients with pain, 28 EM patients without pain, and 29 healthy controls in a Chinese population using a PCR-RFLP assay. *Results:* AA homozygote carriers and A allelic frequencies for -1195 A/G polymorphism in COX-2 gene were significantly increased in EM patients compared with the healthy controls ($p < 0.001$). In addition, further subgroup analysis revealed that the AA genotype and A allele of the -1195 A/G variant were present at a significantly higher frequency in the severe pain group than those in the mild and moderate pain groups. Compared with the controls, the risk of developing EM was 2.86-fold higher in individuals with -1195 AA containing the haplotype, and the risk of developing pain was 2.33-fold higher in EM patients with -1195 AA containing the haplotype. *Conclusions:* These findings suggest that the -1195 A/G on the promoter region of COX-2 gene may increase the risk of pain occurrence in EM women, possibly by affecting the rate of gene expression, especially in patients with the pain phenotype.

Key words: Endometriosis; Pain; Cyclooxygenase-2; Single nucleotide polymorphism.

Introduction

Endometriosis (EM) is a common chronic benign disease in women of reproductive age, but the exact etiology of this disease remains controversial. Pelvic pain is one of the most main clinical symptoms affecting approximately 70% EM patients, and considerably reduces the quality of life in affected women. However, the mechanism of EM-related pain is unclear.

Some current studies [1] suggest that inflammatory response is one of the important factors. Cyclooxygenase-2 (COX-2) is the key rate-limiting enzyme in the conversion of phospholipid arachidonic acid to prostaglandins (PG) via increasing prostaglandin E2. The production of PG catalyzed by COX-2 is involved in inflammation and pain response by multiple pathways in different tissues in the body [2]. In this way, COX-2 may contribute to the progression and continuity of EM. Studies [3, 4] suggested that COX-2 expression was significantly increased in the eutopic endometria, ectopic endometria, and ovarian endometriotic tissue of EM women, and was associated with dysmenorrhea. It implied possible roles of hyperperistalsis in the pathogenesis of EM, particularly in the view of COX-2 and PGE [5]. COX-2 inhibition induces regression of endometrial grafts by suppression of angiogenesis and stimulation of apoptosis [6]. COX-2 inhibitors are believed to be a safe, effective, and low-cost therapy in the management of pelvic pain associated with EM, and were also proposed for use to treatment pelvic pain in the early stage of EM [7]. The single nucleotide polymorphism of COX-2 may play an important role in genetic susceptibility to the development of EM and adenomyosis. So far, there has been little information about the correlation between -1195 A/G gene polymorphism and the risk of pain occurrence in EM patients, and the potential association of -1195 A/G haplotype with different degrees of pain in EM has not been assessed in Chinese women. The primary purpose of this study was to investigate the effect of -1195 A/G polymorphism in COX-2 gene on pain occurrence in endometriotic women and assess the strength of this association (if any) using odds ratios (ORs) with 95% confidence intervals (CIs).

Materials and Methods

Subjects

Included in this study were all patients who underwent open or laparoscopic surgery for pathologically confirmed ovarian endometrial cysts in Dalian Obstetrics and Gynecology Hospital from June 2010 to May 2011. This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Dalian hospital of gynecology and obstetrics. Written informed consent was obtained from all participants.

Classification of clinical phenotypes

According to the presence or absence of dysmenorrhea, 60 EM patients who fulfilled the revised American Fertility Society (r-

Revised manuscript accepted for publication October 8, 2014

Clin. Exp. Obstet. Gynecol. - ISSN: 0390-6663
XLIII, n. 2, 2016
doi: 10.12891/ceog2768.2016

7847050 Canada Inc.
www.irog.net

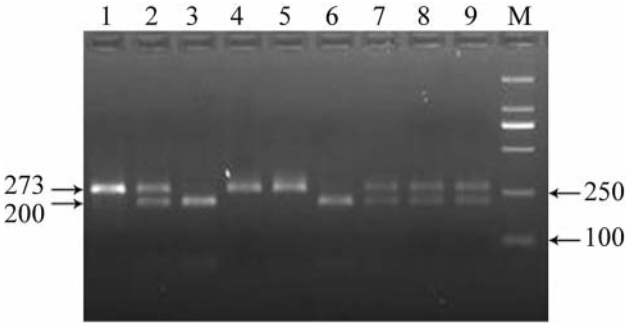

Figure 1. — The electropherogram of -1195 A/G polymorphism genotype in COX-2 gene. Sample 1, 4 and 5 are AA homozygotes (273bp); 3 and 6 lanes are GG homozygotes (200bp and 73bp); 2, 7, 8, and 9 lanes are GA heterozygotes (273bp, 200bp, and 73bp).

AFS) score of Stage III-IV were divided into two groups: 32 in the pain group and 28 patients in the non-pain group. According to the preoperative visual analogue pain scale (VAS) score, the 32 patients in the pain group were further divided into three groups: 1-3 points as light pain, 4-6 as moderate pain, and 7-10 as severe pain. Additional 29 healthy subjects eliminated EM were selected randomly from healthy outpatients who underwent routine physical examination and ultrasound during the same period. The mean age in the EM group was 39.80 ± 2.67 years, which was essentially the same as the control group (37.36 ± 3.04 years). All the included women were Chinese Han nationality with normal menstrual cycles without histories of smoking, chronic pelvic inflammatory disease, hypertension, diabetes, nephropathy, or genetic disease in the families, nor did they administer any hormonal drug at least three months before surgery. Peripheral whole venous blood (two ml) was taken from all the participants, treated with EDTA anticoagulant, and stored at -70 °C until analysis.

Genotype analysis

Genomic DNA was extracted from the peripheral whole blood using the genomic DNA extraction kit. Genotyping was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). The COX-2 gene -1195 A/G PCR primers were the following: 5'-CCC TGA GCA CTA CCC ATGAT-3' (Forward) and 5'-GCC CTT CAT AGG AGATAC TGG-3' (Reverse) [8]. The PCR reaction system was carried out in a final volume of 50 μl by initial denaturation at 98 °C for two minutes, followed by 30 amplification cycles: 98°C during ten seconds for denaturation, 60°C during ten seconds for annealing, and 68°C during 30 seconds for extension, and finally, 68 °C during five minutes for ending extension. The digested products were then electrophoresed on 2% agarose gel stained with ethidium bromide and examined under transillumination (Figure 1). Each gel was assessed independently by two observers unaware of the status of the subjects. If there was any conflict, sample genotyping was repeated.

Sequence analysis of each set of PCR amplification products was performed by cutting gel purified DNA sequencing in the ABI377 automatic sequencing instrument on COX-2 -1195 sites to verify whether the mutation was consistent with gel electrophoresis. -1195 points homozygous were AA and GG, and the heterozygous was GA (Figure 2).

Statistical analysis

For statistical analysis, the observed numbers of each COX-2 -1195 A/G genotype were compared with those expected for population in Hardy-Weinberg equilibrium by using the χ^2 test. The Pearson's χ^2-test was used to analyze the distribution of genotype frequencies between groups. OR and 95% CI were used as the criteria of the association between the genotype and the diseases or pain. A p value < 0.05 was considered statistically significant. Statistical analyses were performed with the statistical package for social sciences (SPSS version 13.0).

Results

The distribution of -1195 A/G genotypes in COX-2 gene showed no significant difference when compared with that predicted from the Hardy-Weinberg genetic equilibrium for either EM patients or controls, indicating that the distribution achieved genetic equilibrium and had group representativeness.

A/G polymorphism in subjects

There was significant difference between EM patients and controls in the distribution of COX-2 -1195 A/G genotypes or in the allelic frequencies (Table 1). The AA genotype and A allele frequency in the EM group were significantly higher than those in the normal control group ($p < 0.05$). The AA genotype and A allele frequency in the endometriotic pain group were significant higher than those in patients without endometriotic pain and the normal control group ($p < 0.05$). In contrast, the GG genotype and G allele frequencies in the normal control group were more prevalent than those in the endometriotic pain group ($p < 0.05$).

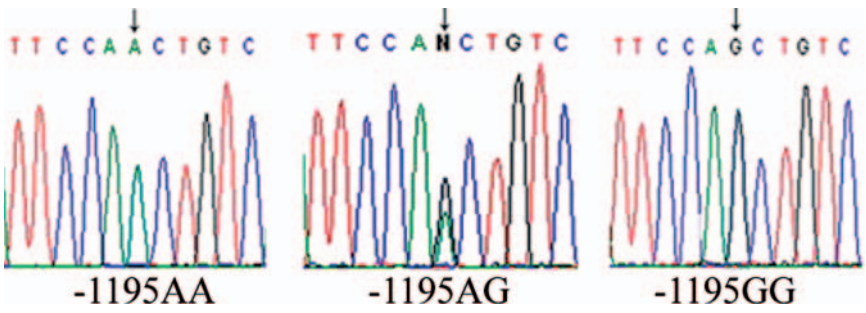

Figure 2. — The single nucleotide polymorphic sequencing result of -1195 A/G in COX-2 promoter region; the arrow indicates a single base change.

Table 1. — *COX-2 gene -1195 A/G genotypes and allelic frequencies in endometriotic pain, non-endometriotic pain, and control groups.*

Group	n	-1195 A/G genotypes (%)			A/G allelic frequencies (%)	
		GG	GA	AA	G	A
EM	60	17(28.33)	24(40.00)	19(31.67)*	58(48.33)	62(51.67)*
Endometriotic pain	32	7(21.88)	11(34.38)	14(43.75)*#	25(39.06)	39(60.94)*#
Non-endometriotic pain	28	10(35.71)	13(46.43)	5(17.86)	33(58.93)	23(41.07)
Control	29	14(48.28)	12(41.38)	3(10.34)	40(68.97)	18(31.03)

Note: *Compared with control group, $p < 0.05$; #compared with non-endometriotic pain group, $p < 0.05$.

Table 2. — *COX-2 gene -1195 A/G genotypes and allelic frequencies in different degrees of pain in EM and control groups.*

Group		n	GG	GA	AA	G allele	A allele
Endometriotic pain	Mild	10	2(20.00)	5(50.00)	3(30.00)	9(45.00)	11(55.00)
	Moderate	10	2(20.00)	4(40.00)	4(40.00)	8(40.00)	12(60.00)
	Severe	12	2(16.67)	3(25.00)	7(58.33)*	7(29.17)	17(70.83)*
Control		29	14(48.28)	12(41.38)	3(10.34)	40(68.97)	18(31.03)

Note: *Compared with control group, $p < 0.05$.

Table 3. — *Correlation analysis of the -1195 AA genotype.*

Group	-1195 AA	-1195 non AA	OR (95%CI)
EM	19 (31.67)	41 (68.33)	2.86 (1.25-7.44)
Control	3 (10.34)	26 (89.66)	
Endometriotic pain	14 (43.75)	18 (56.25)	2.33 (1.09-5.62)
Non-endometriotic pain	5 (17.86)	23 (82.14)	

A/G polymorphism in EM group

The data concerning the COX-2 promoter -1195 A/G polymorphism in different degrees of endometriotic pain are shown in Table 2. The AA genotype and A allele frequency were significantly higher in the severe pain group than those in the mild and moderate pain groups and the normal control group ($p < 0.05$).

The correlation of AA genotype and pain degree

The results of correlation analysis between the -1195 AA genotype and endometriotic pain are shown in Table 3. It was found that the risk of developing EM in individuals carrying two A alleles was 2.86-fold as high as that in the normal control group (95% CI = 1.25 - 7.44). The risk of developing endometriotic pain in EM patients with -1195 AA containing haplotype was 2.33-fold as high as that in the EM patients without pain (95% CI = 1.09 - 5.62).

Discussion

COX is an enzymatic protein existing in two isoforms: COX-1 and COX-2. It is involved in the synthesis of PGE_2 from PGG_2 . COX-2 is an important rate-limiting enzyme in PG synthesis. It is related to the inflammatory response, pain, and fever, and is involved in inflammatory processes and tumor occurrences [9]. The expression of COX-2 was high in ectopic endometrial cells as compared with that in eutopic endometrial cells [10]. The release of PG in ectopic endometrial cells is believed to be involved in the pathogenesis of EM [11]. COX-2 is an inducible enzyme responsible for catalysing the formation of PG and thromboxane in inflammatory settings [12]. Single nucleotide polymorphisms (SNP) of the COX-2 gene may influence gene transcription and PG production in the pelvis.

COX-2 has a variety of gene polymorphisms and the promoter region of a variety of enhancers and transcription control element can activate special transcription factors by altering the transcriptional activity of the gene to control the expression of COX-2. Genetic variation of the promoter region is an important factor to affect the regulation of gene transcription [13].

Several studies [4, 14] have suggested that COX-2 expression is increased in eutopic and ectopic endometrial cells, in ovarian endometriotic tissue of EM patients, and may be related to the pathogenesis of EM. The peritoneal fluid of EM patients can promote the proliferation of endometrial stromal cells, and also induce COX-2 gene expression and enhance PGE_2 secretion in endometrial stromal cells via the MAPK pathway [15]. The COX-2 isoform is an inducible enzyme responsible for PG synthesis in various inflammatory conditions.

COX-2 promoter region -1195 locus is located in the core of the c-Myb transcription factor recognition sequence with G > A single nucleotide polymorphism. The A allele allows the promoter to bind to the transcription factor c-Myb, thus increasing the transcriptional activity of GA or AA genotype, and the susceptibility to disease. A→G mutation significantly reduced COX-2 mRNA transcription [16]. Reports in recent years indicate that COX-2 -1195 G > A single nucleotide polymorphism may play role in increasing susceptibility to EM in China women [17]. The present results showed that the genotype frequencies of -1195 were significantly different in the EM group as compared with controls, which is consistent with the previous reports.

EM is a chronic inflammatory disease characterized by implantation and growth of endometrial tissue outside of the

uterus [18]. COX-2 catalyzes phospholipid arachidonic acid synthesis of PG, which directly activates nociceptors, causing pain, but on the other hand also improves peripheral membrane excitability of primary afferent neurons to reduce feelings nociceptors pain threshold, and increases sensory nerve endings of the sensitivity of the inflammatory mediators. Several studies [19] have shown high expression of COX-2 in ovarian endometrial cyst, eutopic, and ectopic endometrial cells, which may be related to the pathogenesis of EM. The use of COX-2 specific inhibitors is believed to be a safe, effective, and low-cost treatment for EM-associated pelvic pain and may be also proposed in the early stage of EM [20]. However, these results only explain the possible association between COX-2 protein expression and endometriotic pain without confirming whether the COX-2 gene polymorphism is associated with the risk of developing endometriotic pain. The present results showed that there was a significant difference in AA genotype and A allele frequency in COX-2 -1195 between patients with endometriotic pain and those without endometriotic pain and normal controls. In addition, the AA genotype and A allele frequency were significant higher in the severe pain group than those in the controls. -1195 G > A single nucleotide gene polymorphism in COX-2 was related to the risk of developing endometriotic risk, and the A allele indicated a high risk of the occurrence of endometriotic pain.

In summary, COX-2 gene is closely related to the development and progression of EM, implying that COX-2 may be an important factor affecting the susceptibility to EM, and a basic target for the treatment of EM. By far, no study has evaluated the possible relationship between the degree of pain and -1195 A/G polymorphism of COX-2 gene. The A allele of -1195 in COX-2 significantly increased the risk of morbidity and endometriotic pain in the present research. Further study regarding the -1195 G > A gene polymorphism in COX-2 gene is needed to clarify the pathogenesis of endometriotic pain and find the molecular intervention action site on endometriotic pain so as to develop new therapeutic targets for the treatment of EM and endometriotic pain.

Acknowledgments

This study was funded by Dalian Municipal Science and Technology Plan Project (Project number: 20110659).

References

[1] McKinnon B., Bersinger N.A., Wotzkow C., Mueller M.D.: "Endometriosis-associated nerve fibers, peritoneal fluid cytokine concentrations, and pain in endometriotic lesions from different locations". *Fertil. Steril.,* 2012, *97,* 373.

[2] Shahedi K., Lindström S., Zheng S.L., Wiklund F., Adolfsson J., Sun J., *et al.:* "Genetic variation in the COX-2 gene and the association with prostate cancer risk". *Int. J. Cancer.,* 2006, *119,* 668.

[3] Cho S., Park S.H., Choi Y.S., Seo S.K., Kim H.Y., Park K.H., *et al.:* "Expression of cyclooxygenase-2 in eutopic endometrium and ovarian endometriotic tissue in women with severe endometriosis". *Gynecol. Obstet. Invest.,* 2010, *69,* 93.

[4] Matsuzaki S., Canis M., Pouly J.L., Wattiez A., Okamura K., Mage G.: "Cyclooxygenase-2 expression in deep endometriosis and matched eutopic endometrium". *Fertil. Steril.,* 2004, *82,* 1309.

[5] Li X., Gong X., Zhu L., Leng J., Fan Q., Sun D., *et al.:* "Stretch magnitude and frequency-dependent cyclooxygenase 2 and prostaglandin E2 up-regulation in human endometrial stromal cells: possible implications in endometriosis". *Exp. Biol. Med.,* 2012, *237,* 1350.

[6] Laschke M.W., Elitzsch A., Scheuer C., Vollmar B., Menger M.D.: "Selective cyclo-oxygenase-2 inhibition induces regression of autologous endometrial grafts by down-regulation of vascular endothelial growth factor-mediated angiogenesis and stimulation of caspase-3-dependent apoptosis". *Fertil. Steril.,* 2007, *87,* 163.

[7] Kilico I., Kokcu A., Kefeli M., Kandemir B.: "Regression of experimentally induced endometriosis with a new selective cyclooxygenase-2 enzyme inhibitor". *Gynecol. Obstet. Invest.,* 2014, *77,* 35.

[8] Shi J., Misso N.L., Kedda M.A., Horn J., Welch M.D., Duffy D.L., *et al.:* "Cyclooxygenase 2 gene polymorphisms in an Australian population: association of the -1195 G > A promoter polymorphism with mild asthma". *Clin. Exp. Allergy,* 2008, 38, 913.

[9] Moran E.M.: "Epidemiological and clinical aspects of nonsteroidal anti-inflammatory drugs and cancer risks". *J. Environ. Pathol. Toxicol. Oncol.,* 2002, *21,* 193.

[10] Ota H., Igarashi S., Sasaki M., Tanaka T.: "Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis". *Hum. Reprod.,* 2001, *16,* 561.

[11] Chishima F., Hayakawa S., Sugita K., Kinukawa N., Aleemuzzaman S., Nemoto N., *et al.:* "Increased expression of cyclooxygenase-2 in local lesions of endometriosis patients". *Am. J. Reprod. Immunol.,* 2002, *48,* 50.

[12] Gurdol F., Cakmakoglu B., Dasdemir S., Isbilen E., Bekpinar S., Isbir T.: "765 G→C and -1195 A→G Promoter Variants of the Cyclooxygenase-2 Gene Decrease the Risk for Preeclampsia". *Genet. Test. Mol. Biomarkers,* 2012, *16,* 435.

[13] Zhao D., XU D., Zhang X., Wang L., Tan W., Guo Y., *et al.:* "Interaction of cyclooxygenase-2 variants and smoking in pancreatic cancer: a possible role of nucleophosmin". *Gastroenterology,* 2009, *136,* 1659.

[14] Khan K.N., Kitajima M., Fujishita A., Hiraki K., Matsumoto A., Nakashima M., *et al.:* "Pelvic pain in women with ovarian endometrioma is mostly associated with coexisting peritoneal lesions". *Hum. Reprod.,* 2013, *28,* 109.

[15] Liu Y., Hu J., Shen W., Wang J., Chen C., Han J., *et al.:* "Peritoneal fluid of patients with endometriosis promotes proliferation of endometrial stromal cells and induces COX-2 expression". *Fertil. Steril.,* 2011, *95,* 1836.

[16] Zhang X., Miao X., Tan W., Ning B., Liu Z., Hong Y., *et al.:* "Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of esophageal cancer". *Gastroenterology,* 2005, *129,* 565.

[17] Wang Y.F., Qu Y.E., Yang M.: "Study on the relationship between polymorphism of the COX-2 gene and endometriosis and adenomyosis". *Shan Dong Medical Journal.,* 2007, *47,* 22.

[18] Gan X.L., Lin Y.H., Zhang Y., Yu T.H., Hu L.N.: "Association of an interleukin-16 gene polymorphism with the risk and pain phenotype of endometriosis". *DNA Cell. Biol.,* 2010, *29,* 663.

[19] Banu S.K., Lee J., Speights V.O. Jr., Starzinski-Powitz A., Arosh J.A.: "Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms". *Endocrinology,* 2008, *149,* 1180.

[20] Cobellis L., Razzi S., De Simone S., Sartini A., Fava A., Danero S., *et al.:* "The treatment with a COX-2 specific inhibitor is effective in the management of pain related to endometriosis". *Eur. J. Obstet. Gynecol. Reprod. Biol.,* 2004, *116,* 100.

Address reprint requests to:
H. WANG, M.D.
Department of Gynecology
Dalian Hospital of Gynecology and Obstetrics
No.1 Dunhuang Road
Dalian city 116033
Liao Ning Province (China)
e-mail: hualiwangcn@163.com