Evaluation of *KRAS* Gene Expression and LCS6 Variant in Genomic and Cell-Free DNA of Iranian Women With Endometriosis

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Abstract

Since the activation of *KRAS* results in de novo endometriosis in mice, *KRAS* is regarded as a crucial gene in ectopic endometrial implantation. Recently, it has been reported that 31% of women with endometriosis have *KRAS* let-7 complementary binding site 6 single-nucleotide polymorphism (LCS6 SNP). This study addresses the correlation between *KRAS* LCS6 SNP and endometriosis in a case-control study. To detect probable somatic mutation in ectopic endometrial tissue, we evaluated LCS6 SNP in cell-free DNA samples. Quantitative real-time reverse transcription-polymerase chain reaction was performed to determine the expression of *KRAS* transcripts in eutopic endometrial tissue. Our results suggest that the variant is not associated with the development of endometriosis in Iranian women. We observed higher levels of *KRAS* messenger RNA (mRNA) expression in eutopic endometrium of patients with endometriosis compared to controls. Although, the *KRAS* LCS6 is neither constitutional nor somatic biomarker for endometriosis, increased expression ratio of *KRAS* mRNA indicates its role in the implantation of endometrial tissue outside the uterine cavity.

Keywords

endometriosis, *KRAS* gene, let-7 microRNA, cell-free DNA, single-nucleotide polymorphism

Introduction

Endometriosis is one of the most common benign gynecological diseases in women of reproductive age. The disease is defined as the implantation of endometrial glands and stroma outside the uterus, which can be found on the surfaces of ovaries and other pelvic cavity organs.¹ The main clinical symptoms of the disease are dysmenorrhea, dyspareunia, pelvic pain, and infertility.² Since the diagnosis of endometriosis is highly dependent on invasive surgical methods, the precise prevalence of the disease remains unknown. However, estimates show that 10% to 15% of women in reproductive age are affected.³

Despite extensive investigations, the molecular etiology of endometriosis is still unclear. The role of genetic predisposing factors was suggested by the observation of familial occurrence of endometriosis. It has been identified that the relatives of the patients have up to 10-fold higher risk of the disease.⁴ Various genes have been studied in endometriosis, including the genes that are involved in peritoneal surface adhesion, invasion, proliferation, vessels formation, inflammatory responses, and signal transduction.⁵ Specifically, activation of *KRAS* gene resulted in de novo endometriosis in mice, indicating the importance of Rat sarcoma (RAS) pathway in the initiation of invasions.⁶ Eutopic endometrial

gene expression profile in experimental endometriosis of baboon provided additional insight into the underlying role of *KRAS*.⁷ However, molecular analysis in human endometriosis failed to detect any mutation in the coding region of the *KRAS* gene.^{8,9}

A high frequency of *KRAS* 3'UTR variant rs61764370, which is located at microRNA (miRNA) let-7 complementary binding site 6 (LCS6), has recently been reported in endometriosis (31% of women with endometriosis as opposed to 5% of a diverse control population).¹⁰ Since the frequency of this allele differs across populations,¹¹ we assessed the LCS6 single-nucleotide polymorphism (SNP) distribution in apparently healthy Iranian people (comprising men and women) without any consideration of

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endometriosis. Subsequently, a case–control design was applied to evaluate the spread of the variant in histopathologically confirmed endometriosis and a control group composed of females who were surgically proven to have no endometriosis. Assuming that LCS6 SNP would occur somatically, we evaluated plasma cell-free DNA (CFDNA) as the representative of ectopic endometrial tissue.

Methods

Patients

Considering that the *KRAS* LCS6 SNP is a rare variant, 100 randomly selected people from distinct Iranian ethnic groups were genetically tested to estimate the allele frequency in our population.

In the next step, we focused on the association of *KRAS* LCS6 SNP and endometriosis in a case–control study. The participants were referred to Firozgar Hospital in Tehran from March 2013 to February 2014. Fifty-eight patients with endometriosis were selected based on the direct observation of the ectopic endometrial tissue via laparotomy or laparoscopy and histological confirmation of the diagnosis. Surgical staging of the lesions revealed mild (stage II) to moderate (stage III) endometriosis according to the revised American Society for Reproductive Medicine score. The main subtype was ovarian (75.6%) along with ovarian/fallopian tubes (8.1%), ovarian/abdominal wall (5.4%), ovarian/omentum (2.7%), and vaginal/cervical (8.2%). The patients had undergone the surgery to resect endometriotic ovarian cysts and nodules. There was no recurrent endometriosis requiring surgical intervention included in the study.

The control group ($n = 60$) was composed of the females of reproductive age who had undergone laparotomy or laparoscopy for tubal ligation or other benign gynecological diseases. The participants were interviewed based on a structured questionnaire, which covered questions regarding demographics, body mass index (BMI), pregnancy history, contraceptive methods, menstrual regularity, abnormal bleeding, hormonal treatment, prior medical and surgical conditions, and family history of endometriosis. The study procedures were approved by the ethical committee of Tarbait Modares University.

Genomic and Cell-Free DNA Isolation

Peripheral blood samples (5 mL) were collected in EDTA containing tubes ahead of surgery. The plasma was immediately separated by centrifugation at 2000g for 10 minutes and stored at -20°C . Genomic DNA was isolated from peripheral blood leukocytes using the DIAtom DNA Extraction kit (Gene Fanavaran Co, Iran). Nucleo Spin Plasma XS Kit (MachereyNagel, BaharTashkhisTeb, Iran) was used to isolate CFDNA from 400 μL of the plasma samples according to the manufacturer's instructions.

Genomic and Cell-Free DNA Genotyping

Identification of *KRAS* LCS6 polymorphic variant (rs61764370, T to G) was carried out by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Polymerase chain reaction was performed using 10 pmol of forward (5'CTGCCCCAATCCATTAGCCAC3') and reverse (5'CACAGAGACCAAAACCCCTTCT3') primers and 50 ng of genomic DNA added to 20 μL PCR mastermix (Amplicon, Pishgam, Iran). The thermal cycling conditions were 95°C for 5 minutes, 35 cycles composed of 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 40 seconds, and a final extension of 10 minutes at 72°C . To further amplify the product of CFDNA PCR, additional nested PCR reaction was established using the same primers on 4 μL of the product of the first PCR round.

After the amplification of desired fragments (699 bp), the PCR products were digested by endonuclease PfiI (TfiI) enzyme (Thermo Scientific, Gene Fanavaran Co, Iran). The PCR products containing *KRAS* LCS6T allele were cleaved into 313-, 135-, and 251-bp fragments, whereas PCR products containing the G allele were cleaved into 386- and 313-bp fragments. The DNA fragments were separated and visualized by electrophoresis on 2% agarose gel containing Green Viewerdye (Arya Tous Biotech, Iran). Randomly selected PCR products were sequenced to confirm the results of PCR–RFLP analysis.

Eutopic Endometrium RNA Isolation

Tissue samples of eutopic endometrium were transferred into liquid nitrogen immediately after collection. Total RNA was isolated using GeneJET RNA Purification Kit as recommended by the manufacturer's instructions (Fermentas, Genefanavar, Iran). The concentration of the purified RNA was determined by NanoDrop 2000C (Thermo Scientific, Tuba Negin, Iran). Total RNA (up to 500 ng) was subjected to complementary DNA (cDNA) synthesis using PrimeScript RT reagent kit (Takara, Aminsan, Iran) according to the manufacturer's directions.

Quantitative Real-Time Reverse Transcription PCR Analysis of *KRAS* Messenger RNA

Quantitative real-time PCR reactions were carried out using SYBR Premix Ex TaqII (Takara, Aminsan, Iran) and *KRAS*-specific primers as described previously.¹⁰ The experiments were performed in duplicate on a Rotor-Gene Q instrument (QIAGEN GmbH, Germany). The PCR amplification program was as follows: 30 seconds at 95°C ; 35 cycles of 5 seconds at 95°C , 30 seconds at 59°C , and 40 seconds at 72°C . Melting curves were generated after each run to verify the amplification of the desired amplicons, which was shown by the presence of a single specific peak and the absence of primer dimers. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) messenger RNA (mRNA) was used to normalize *KRAS* mRNA expression for differences

Table 1. Clinical Characteristics of the Study Participants Including Age, BMI, and Distribution of the Parity in Each Group.

Characteristic	Endometriosis (n = 58)	Controls (n = 60)	P Value
Age, mean $\pm$ SD	36.66 $\pm$ 8.69	36.09 $\pm$ 7.87	.775
Body mass index, mean $\pm$ SD	26.02 $\pm$ 4.98	27.30 $\pm$ 4.04	.157
Gravidity, mean $\pm$ SD	2.09 $\pm$ 1.83	3.25 $\pm$ 1.89	.004
Parity, mean $\pm$ SD	1.51 $\pm$ 1.43	2.79 $\pm$ 1.79	.000

Abbreviations: BMI, body mass index; SD, standard deviation.

in RNA input. The PCR efficiency of *KRAS* and *GAPDH* genes were determined by 1:4 serial dilutions of cDNA as the PCR template. The Ct values obtained from the serially diluted reactions were plotted against their concentration log in order to generate standard curves. The efficiency of each reaction was determined using the equation $E = 10^{[-1/\text{slope}]}$. Relative *KRAS* gene expression was calculated as the ratio of the gene expression in test sample relative to the normal calibrator samples ($\Delta\Delta C_t$ method). The formula ratio = $2^{-\Delta\Delta C_t}$ was used to estimate relative gene expression changes where $\Delta\Delta C_t = \Delta C_t$ (endometriosis) - ΔC_t (mean normal).

Statistics

Power and sample size of the study were determined using EPI info (V.7.0.9.34) software for genetic epidemiology studies. The analysis of data was performed by SPSS software (V.18). The *t* test was used to evaluate statistical significance of the differences in comparative analyses. The differences in genotype frequencies between the study groups were assessed by standard chi-square, the odds ratio (OR), and 95% confidence intervals (CIs). *P* value < .05 was considered statistically significant.

Results

Clinical Characteristics of the Patients

The mean age, mean BMI, and the distribution of the parity in each group are described in Table 1. The assessment of menstrual history showed that the patients with endometriosis experienced significantly more severe dysmenorrhea than controls ($P < .013$, 95% CI 1.23-8.93, Pearson χ^2 5.94). In contrary to expectation, we did not observe significant correlation between endometriosis and infertility. This arises as a result of the few infertile cases that participated in the study. However, we observed significant differences between the endometriosis and normal groups in terms of gravidity ($P = .004$) and parity ($P = .000$). These conditions are probably related to the endometriosis subfertility status (Table 1). The data also revealed no significant link between endometriosis and abnormal menstrual bleeding, contraceptive methods, menstrual regularity, and family history.

Figure 1. A, Restriction digestion of polymerase chain reaction (PCR) products demonstrating the different genotypes patterns of *KRAS* LCS6 T > G polymorphism. 1, 100-bp ladder; 2, the 699-bp undigested PCR product; 3, 4, 5, 6, 7, 8, 10, and 11, homozygote TT; and 9, heterozygote TG genotypes. B, Up: sequencing result of an individual with TG genotype and down: sequencing result of an individual with TT genotype.

As contraceptive methods (eg, tubal ligation, pills, barriers) and hormonal treatment influence the study variables, we adjusted the analyses for these parameters. However, the results remained unchanged, suggesting that the analysis is not markedly affected by confounding factors.

Genomic DNA Genotyping

As shown in Figure 1A, the desired digested fragments of *KRAS* LCS6 variant were revealed by gel electrophoresis. Furthermore, the sequencing of the PCR products confirmed the PCR-RFLP results (Figure 1B).

Minor allele frequency of rs61764370 variant was 0.03 (6 of 200) in Iranian general adult population. Among the endometriosis group (n = 58), 1 (1.72%) patient was found to carry the SNP, whereas 4 (6.66%) participants in the control group had the allele in heterozygous state. To analyze the correlation between *KRAS* LCS6 SNP and the

Figure 2. Melting curve analysis of the real-time polymerase chain reaction (PCR) experiments for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *KRAS* gene expression study. The dissociation stage of the real-time PCR experiment was performed by ramping temperature from 60°C to 95°C. The presence of desired amplicons of each gene was indicated by a specific single peak in the melting curve graph. Nontemplate controls (NTC) were also indicated as curves with no specific amplification.

endometriosis risk, the TT genotype was given as the reference genotype. Then, the only other genotype found (TG) was compared with the reference genotype. A decreased risk of endometriosis was observed for carriers of *KRAS* LCS6 SNP G allele with the OR of 0.24 [95% CI 0.2-2.2]. Confirmation of the protective effect of the variant requires analysis with higher statistical power in a larger study. There was no association between rs61764370 SNP TG genotypes and other gynecological symptoms or reproductive status of the patients.

Cell-Free DNA Genotyping

To check the probable somatic mutations, the allele frequency of the SNP was measured in CFDNA of both case and control groups. The genotype of genomic DNA and CFDNA were perfectly matched in each patient, indicating that *KRAS* LCS6 SNP somatic mutations are not common in endometriosis. To further validate the CFDNA analysis, we selected an expectant family with TG genotype father, and TT genotype mother, who might carry a fetus with TG genotype. Blood sample was collected from the full-term pregnant mother. Interestingly, we found TG genotype in the CFDNA of the mother who had been genotyped TT in genomic DNA. The results were confirmed by sequencing analysis of *KRAS* LCS6 SNP in the newborn infant.

Quantitative Real-Time PCR Analysis of *KRAS* mRNA

Polymerase chain reaction amplification of *KRAS* and *GAPDH* mRNAs was successfully performed as revealed by melting curves analysis of the products. The amplification of the desired amplicons was shown by the presence of a single specific peak for each gene (Figure 2). The stability of *GAPDH* mRNA expression in eutopic endometrial tissue from patients

Figure 3. Relative expression levels of *KRAS* messenger RNA (mRNA) in the endometriosis and normal groups.

with endometriosis and controls was verified by comparing the Ct values in the 2 groups. Mean Ct value of *GAPDH* was 12.33 ± 1.4 and 11.93 ± 1.3 in endometriosis and normal samples, respectively ($P = .24$). Analysis of *KRAS* gene expression in eutopic endometrium was carried out by $\Delta\Delta C_T$ method. We found that the expression levels of mRNA in the endometriosis group (relative expression = 1.17 ± 0.42) were significantly higher ($P = .041$) compared to the nonendometriosis group (relative expression = 0.74 ± 0.20 ; Figure 3). To evaluate the effect of menstrual cycle phases on *KRAS* expression, the data were weighted to the phases, and no significant correlation was observed ($P = .30$).

Discussion

Let-7 miRNAs, comprising let-7a-g and let-7i, are involved in the regulation of *KRAS* gene expression. The expression adjustment is influenced by the ability of let-7 to properly attach to its binding sites on *KRAS* mRNA. Thus, SNPs located within the miRNA-binding site is considered as mRNA and protein expression modifiers. Grechukhina and colleagues presented *KRAS* LCS6 SNP, rs61764370, as a novel gene mutation associated with endometriosis.¹⁰ They were the first to introduce this variant allele as a genetic marker for endometriosis risk assessment and early diagnosis.

KRAS LCS6 SNP has been suggested as a biomarker for epithelial ovarian cancer in a study composed of 3 independent cohorts.¹² However, further large investigation in *BRCA1* carrier or noncarrier patients with ovarian cancer did not provide any evidence to support this finding.¹³ In addition, *KRAS* LCS6 SNP did not appear to predict increased risk of sporadic and familial breast cancer except in postmenopausal patients with high-grade tumor, who had

received hormone replacement therapy.¹⁴ A recent publication found no evidence of association between the *KRAS* LCS6 SNP and overall endometrial cancer risk, although it was introduced as a genetic marker for the risk of estrogen insensitive type 2 endometrial cancers.¹⁵

An earlier investigation reported that the rs61764370 allele frequency appeared to be ethnically different, by the rate of 0.03 in Asian.¹¹ We calculated the minor allele frequency of 0.03 in Iranian general adult population, which was in agreement with the expected value. To investigate the possible correlation between endometriosis and *KRAS* LCS6 SNP, a case-control study was conducted in Iranian population. This study shows no significant link between rs61764370 and endometriosis. These findings proposed that *KRAS* LCS6 SNP is not a functional marker to identify patients with high-risk endometriosis in certain population. Based on genome-wide association study, the same finding was reported by a European report.¹⁶ In addition to geographical variations, other contributing factors such as probable somatic alteration of *KRAS* LCS6 SNP in endometriosis tissue, help explain the controversy.

By evaluating CFDNA, we analyzed the circulating somatic DNA released from ectopic endometrium. It has been demonstrated that concentrations of CFDNA in plasma and serum could be used to monitor a variety of noninvasive malignancies and benign proliferative conditions including endometriosis.^{17,18} Since obtaining adequate ectopic tissue in mild to moderate endometriosis is an obstacle for molecular analysis, CFDNA provides an appropriate alternative basis for mutation detection. In this study, for the first time, we used CFDNA as a representative of ectopic endometrial tissues to elucidate possible somatic variation in *KRAS* LCS6 SNP. However, no T to G alteration was observed when CFDNA was compared to corresponding genomic DNA. It should be noted that CFDNA originated from healthy, inflamed, or diseased tissue may increase the ratio of non-LCS6/LCS6 DNA in the plasma. This would limit the sensitivity of conventional PCR-RFLP to detect a heterozygote allele. Real-time PCR or other advanced molecular techniques could overcome this limitation. We concluded that the accuracy of CFDNA as a somatic indicator of the endometriosis needs to be further verified within the context of detailed molecular studies with sufficient power.

As a strategy to discover *KRAS* gene expression pattern in eutopic endometrium of patients with endometriosis, we conducted a real-time reverse transcription PCR assay. Increased *KRAS* gene expression was evident in endometriosis, independent of the LCS6 SNP.

KRAS protein serves as a molecular switch for cell proliferation and is mostly linked to tumor growth and differentiation.¹⁹ The *KRAS* LCS6 SNP has been shown to be associated with altered gene expression. By luciferase assay in A549 lung cancer cell line, Chin and colleagues found a significant increase in *KRAS* mRNA harboring LCS6 SNP.¹¹ Grechukhina et al reported that *KRAS* LCS6

SNP facilitates a higher proliferation and invasion rate of endometrial cells. They also observed higher *KRAS* mRNA levels even in the endometriosis tissue lacking LCS6 SNP.¹⁰

In conclusion, despite a declaration of *KRAS* LCS6 SNP as the first described genetic marker of endometriosis risk, we found no statistically significant difference in allele frequency distribution between the cases and the controls. Furthermore, the presented results indicate that overexpression of *KRAS* gene presumably impacts endometrial cell proliferation and incursion toward endometriosis pathology.

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Declaration of Conflicting Interests

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References

1. Benagiano G, Brosens I. The history of endometriosis: identifying the disease. *Hum Reprod*. 1991;6(7):963-968.
2. Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364(9447):1789-1799.
3. Allaire C. Endometriosis and infertility: a review. *J Reprod Med*. 2006;51(3):164-168.
4. Matalliotakis IM, Arici A, Cakmak H, et al. Familial aggregation of endometriosis in the Yale Series. *Arch Gynecol Obstet*. 2008;278(6):507-511.
5. Kao LC, Germeyer A, Tulac S, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology*. 2003;144(7):2870-2881.
6. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T. Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med*. 2005;11(1):63-70.
7. Afshar Y, Hastings J, Roqueiro D, Jeong JW, Giudice LC, Fazleabas AT. Changes in eutopic endometrial gene expression during the progression of experimental endometriosis in the baboon, *Papio anubis*. *Biol Reprod*. 2013;88(2):44.
8. Vercellini P, Trecca D, Oldani S, Fracchiolla NS, Neri A, Crosignani PG. Analysis of p53 and ras gene mutations in endometriosis. *Gynecol Obstet Invest*. 1994;38(1):70-71.
9. Zhao ZZ, Nyholt DR, Le L, et al. *KRAS* variation and risk of endometriosis. *Mol Hum Reprod*. 2006;12(11):671-676.
10. Grechukhina O, Petracco R, Popkhadze S, et al. A polymorphism in a let-7 microRNA binding site of *KRAS* in women with endometriosis. *EMBO Mol Med*. 2012;4(3):206-217.

11. Chin LJ, Ratner E, Leng S, et al. A SNP in a let-7 microRNA complementary site in the KRAS 3' untranslated region increases non-small cell lung cancer risk. *Cancer Res.* 2008; 68(20):8535-8540.
12. Ratner E, Lu L, Boeke M, et al. A KRAS-variant in ovarian cancer acts as a genetic marker of cancer risk. *Cancer Res.* 2010;70(16): 6509-6515.
13. Pharoah PD, Palmieri RT, Ramus SJ, et al. The role of KRAS rs61764370 in invasive epithelial ovarian cancer: implications for clinical testing. *Clin Cancer Res.* 2011;17(11): 3742-3750.
14. Cerne JZ, Stegel V, Gersak K, Novakovic S. KRAS rs61764370 is associated with HER2-overexpressed and poorly-differentiated breast cancer in hormone replacement therapy users: a case control study. *BMC Cancer.* 2012;12:105.
15. Lee LJ, Ratner E, Uduman M, et al. The KRAS-variant and miRNA expression in RTOG Endometrial Cancer Clinical Trials 9708 and 9905. *PLoS One.* 2014;9(4):e94167.
16. Luong HT, Nyholt DR, Painter JN, et al. No evidence for genetic association with the let-7 microRNA-binding site or other common KRAS variants in risk of endometriosis. *Hum Reprod.* 2012;27(12):3616-3621.
17. Zachariah R, Schmid S, Radpour R, et al. Circulating cell-free DNA as a potential biomarker for minimal and mild endometriosis. *Reprod Biomed Online.* 2009;18(3):407-411.
18. Vlassov VV, Laktionov PP, Rykova EY. Circulating nucleic acids as a potential source for cancer biomarkers. *Curr Mol Med.* 2010; 10(2):142-165.
19. Vakiani E, Solit DB. KRAS and BRAF: drug targets and predictive biomarkers. *J Pathol.* 2011;223(2):219-229.