Lack of an Association between a Polymorphism in the KRAS 3' Untranslated Region (rs61764370) and Endometriosis in a Large European Case-Control Study

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Keywords

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Abstract

Background: Endometriosis is a common disorder that affects 6–10% of reproductive age women. In a previous study, we demonstrated that a polymorphism in *let-7* microRNA-binding site in the 3' untranslated region of the *KRAS* gene was found in 31% of subjects with endometriosis resistant to medical therapy. This polymorphism was now tested in a large, case-control study. **Methods:** Peripheral blood or peritoneal biopsies from 2,077 European subjects with or without endometriosis and known infertility were tested for the presence of the variant allele using polymerase chain reaction. **Results:** Histologically proven endometriosis was found in 1,140 subjects, while 937 subjects were disease free. Variant allele carrier rates in subjects with and without endometriosis were 15.7 and 15.1%, respectively. No association between the variant *KRAS* allele and stage of the disease, age at surgery, body mass index, or type of infertility was identified. **Conclusion:** A germ-line single-nucleotide polymorphism in the *let-7* microRNA-binding site of the *KRAS* gene was not associated with sporadic endometriosis in an infer-

tile Caucasian population in this large case-control study. However, it remains possible that this gene variant may be a marker of treatment resistance. Further studies on the role of this polymorphism in endometriosis are needed.

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Introduction

Endometriosis is characterized by the presence of endometrial glands and stroma outside of endometrial cavity. The condition affects 6–10% of reproductive age women and is associated with chronic pelvic pain, dysmenorrhea, abnormal uterine bleeding, and infertility/subfertility [1–3]. Endometriosis is found in up to 50% of patients with infertility [1, 4]. Presentation of the disease may vary from nearly absent symptoms when the diagnosis is made incidentally during pelvic or abdominal surgery for other indications, to debilitating disease requiring multiple surgical interventions and associated with significant impairment of a woman's quality of life. Due

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to various and sometimes confusing clinical presentations, the diagnosis is commonly delayed by many years [5, 6].

Although multiple medications are being used for endometriosis-related symptoms (oral contraceptives, progestins, gonadotropin-releasing hormone agonists), medical therapy is frequently ineffective, whereas surgical management results in high recurrence of the disease (6–67%), requiring repeat surgery in 36% of cases [7–10]. Such heterogeneity of presentations and discrepancies in treatment results may be related to disease variants, driven by distinct molecular phenotypes. A better understanding of these disease variants is needed to be able to individualize treatment and achieve better outcomes.

Although genetic predisposition to endometriosis has been proposed, specific genes responsible for endometriosis are yet to be identified [11]. In the only in vivo model of spontaneous murine endometriosis (Dinulescu et al. [12]), the activation of an oncogenic *KRAS* gene in the murine ovarian surface epithelium resulted in the de novo formation of lesions with endometriotic morphology and suggested that *RAS* pathway activation may play a role in the development of endometriosis in humans. However, this observation has not been confirmed so far in women by mutational analysis of the coding regions of human *KRAS* gene [13–15].

MicroRNAs, small noncoding RNA molecules, are known to be powerful posttranscriptional regulators of gene expression [16]. They bind to the 3' untranslated region (UTR) on the target messenger RNA (mRNA) and ultimately result in decreased protein translation. The *let-7* microRNA family is known to suppress translation of *KRAS* [17]. A specific single-nucleotide polymorphism (SNP) in the *KRAS* gene rs61764370, in which a single-nucleotide substitution T (wild-type allele) G (variant allele) occurs in a *let-7* microRNA complementary site 6 (LCS6), was shown to be a risk factor for certain cancers, including nonsmall cell lung cancer [18], ovarian cancer in specific populations [19], and triple negative breast cancer [20]. Importantly, this polymorphism, now referred to as the *KRAS*-variant, was first suspected to be prognostic of poor outcome in cancer partly due to its enrichment in patients with metastatic disease [21–23]. However, it has now been definitively shown that instead, the *KRAS*-variant is a predictive biomarker of treatment response across many cancers, including ovarian cancer [24], head and neck cancer [22], colon cancer [25], and nonsmall cell lung cancer [26], with some standard of care treatments working well, and others poorly, allowing the development of advanced and recurrent disease.

Based on the extensive evidence for *RAS* pathway activation/alterations in endometriosis, we previously tested the hypothesis that *KRAS*-variant might be implicated in endometriosis [27]. In our study we found that 31% (41 out of 132) of analyzed subjects with histologically proven endometriosis carried the *KRAS*-variant allele, a frequency much higher than that found in the general population [18, 27]. Furthermore, in this study, endometriosis in women with the *KRAS*-variant was found to be biologically unique with higher proliferation rate and progesterone resistance. However, several studies by other groups failed to reveal an association between this variant and endometriosis [28]. In 2010, a genome-wide association (GWA) study in a Japanese population by Uno et al. [29] demonstrated a strong association between endometriosis and SNPs in *CDKN2BAS* (on chromosome 9p21) and on chromosome 1p36 which contained *WNT4*, but not with a SNP linked to the *KRAS*-variant rs61764370. Subsequently, Painter et al. [30] in 2011 identified association of an intergenic SNP on chromosome 7p15 with endometriosis in a large European cohort. In 2012, Luong et al. [31] analyzed GWA data that included 3,194 surgically confirmed endometriosis cases and 7,060 controls specifically for 2 SNPs that were known to be highly correlated with the rs61764370, followed by genotyping and concluded that there was no association between endometriosis and the *KRAS*-variant allele. Another GWAS by Albertsen et al. [32] demonstrated involvement of the area around *WNT4* including 2 new genes *HSPC157* and *CDC42* in the development of endometriosis in European population. Using a Taqman assay, these authors were unable to confirm an association between the *KRAS*-variant allele rs61764370 and endometriosis in 1,123 cases and 832 controls.

In view of these conflicting results, we decided to assess the frequency of the variant *KRAS*-allele in patients with endometriosis in a large case-control study using the same method that was applied in our previous study. We thus sought to determine if the *KRAS*-variant allele is enriched in women with surgically documented endometriosis or, alternatively, instead might be a marker of treatment resistant endometriosis – a uniform characteristic of population included in our prior study.

Materials and Methods

Sample and Data Collection

A total of 2,077 women who had undergone a laparoscopy for subfertility with or without pain from January 1993 to February 2010 were included based on the following inclusion criteria: lapa-

roscopic and histologically proven presence of endometriosis or laparoscopic absence of endometriosis. All consecutive subjects undergoing surgery for subfertility were enrolled prospectively, and written informed consent was obtained from all women prior to the surgery. Peripheral blood samples or intraoperative peritoneal biopsies were collected. Women with endometriosis had either minimal (stage I), mild (stage II), moderate (stage III), or severe (stage IV) disease, staged according to the revised American Fertility Society (AFS) classification system [33]. In cases, where endometriosis was diagnosed, surgical resection or ablation was performed. The study was approved by the Commission for Medical Ethics of the Leuven University Hospital, Belgium, and the Regional Ethics Committee, Karolinska Institute, Stockholm, Sweden.

DNA Extraction

As previously described (Sundqvist et al. [34]), DNA purified from EDTA-stabilized whole peripheral blood was collected for routine molecular diagnostic tests at the Centre for Human Genetics of University Hospitals, Leuven, Belgium. Various purification methods were used: Chemagic DNA blood special kit (Chemagen MSM I, PerkinElmer Chemagen Technologies GmbH, Baesweiler, Germany), Auto Pure LS Puregene chemistry (Qiagen, Venlo, The Netherlands). The choice of the extraction method was based on the available amount of blood and on the type of required molecular diagnostic test. DNA from peritoneal biopsies was purified with QIAGEN QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNA concentration was measured with NanoDrop 1,000 (Thermo Scientific, Wilmington, DE, USA). For tissue samples with 260:280 ratios <1.5, a DNA clean-up/purification with phenol: chloroform (P 2069, Sigma-Aldrich, Saint Louis, MO, USA) was performed. DNA concentration was measured using Victor (PerkinElmer, MA, USA).

Genetic Testing

For high-throughput genotyping, the isolated DNA samples were amplified using TaqMan polymerase chain reaction assays designed specifically to identify the T or G allele of the LCS6 SNP of *KRAS* gene (Applied Biosystems, Foster City, CA, USA) as was described previously [18]. The work was performed at MiraDx, in their CLIA approved laboratory, using their *KRAS*-variant allele assay.

Statistical Analysis

Statistical analysis of the data was performed using IBM SPSS Statistical Data Editor. Chi-square test was used to analyze categorical data (frequencies of different alleles, types/causes of infertility, stages of endometriosis), while student *t* test and 1-way ANOVA were used to compare interval data (body mass index [BMI], ages, AFS scores). Statistical significance was determined as $p < 0.05$.

Results

Group Characteristics

A total of 2,077 subjects were analyzed for the *KRAS*-variant, all of which underwent laparoscopic surgery for

infertility or subfertility. About 90% of subjects included in the study were European Caucasians. For most patients, this was their initial surgery indicated by infertility/subfertility. Totally, 1,140 women had histologically proven endometriosis, while 937 had no laparoscopic evidence of the disease. Infertility was documented in the medical records of 1,071/1,140 and in 889/937 in the groups with and without endometriosis, respectively. Subjects were noted to be of older age at the time of the surgery in the group without endometriosis (31.4 vs. 32.1 year old, $p = 0.0036$; Table 1). Subjects with endometriosis were more likely to have primary rather than secondary infertility compared to those without endometriosis (76 vs. 62%, $p = 0.0001$). Subjects without endometriosis were more likely to be smokers (14.4 vs. 10.7%, $p = 0.011$). In cases where the cause of infertility/subfertility was known, the groups with endometriosis versus no endometriosis were statistically different based on this stratification. Ovulation, implantation, tubal, and genetic problems were identified in 9.5, 1.4, 5.6, and 0.3%, respectively, in the group with a known endometriosis and 20.2, 3.5, 24.9, and 1% in the group without laparoscopic evidence of endometriosis ($p < 0.00001$). In 83% of cases with endometriosis, the cause of infertility was categorized as “unexplained” versus 50% of such cases in the group without endometriosis (Table 1). “Unexplained” infertility in patients with endometriosis was frequently ascribed to endometriosis itself, however, still classified as unexplained. In the group with endometriosis, the stage of the disease stratification was as follows: Stage I – 34.4%, Stage II – 21.3%, Stage III – 16.5%, and Stage IV – 26.5%. About 1.1% of patients with endometriosis did not have an assigned stage in their chart. Mean AFS score for all endometriosis patients was 29 ± 33.6 . Subjects who smoke daily were more likely to have tubal factor infertility than non-smokers.

Frequency of G Versus T Alleles in LCS6 on 3'UTR of the KRAS Gene

In the entire tested population ($n = 2,077$), the TT genotype (aka wild type) was found in 84.5% ($n = 1,756$), TG in 14.8% ($n = 308$), and GG in 0.6% ($n = 13$) of cases. The frequency of the G allele (variant) was thus calculated as 8.04% ($[(\text{number of G alleles in heterozygotes} + \text{number of G alleles in homozygotes} \times 2)] / \text{total number of T and G alleles} = [308 + 13 \times 2] / [2,077 \times 2]$). Due to a very low frequency of cases homozygous for the G allele ($n = 13$), we considered heterozygous cases with TG genotype to be positive for a variant allele and cases homozygous for G allele (GG genotype) were added to this group in all subsequent

Table 1. Comparison of the groups with and without endometriosis

	Endometriosis	No endometriosis	<i>p</i> value
Number	1,140	937	
BMI, mean (SD)	23.1 (4.19)	23.9 (4.53)	0.001
Infertility – any, <i>n</i> (%)	1,071 (93.9)	889 (94.8)	0.39
Primary, <i>n</i> (%)	822 (72.1)	556 (59.3)	0.0001
Secondary, <i>n</i> (%)	249 (21.8)	333 (35.5)	0.0001
Anovulation	102 (9.5)	180 (20.2)	0.00001
Implantation	15 (1.4)	32 (3.5)	0.00001
Tubal	60 (5.6)	222 (24.9)	
Genetic	4 (0.3)	9 (1)	
Unexplained	890 (83)	446 (50)	
Smoking, <i>n</i> (%)	123 (10.7)	136 (14.5)	0.0112
Age at surgery, mean (SD)	31.4 (4.3)	32.1 (4.8)	0.0036

BMI, body mass index.

Table 2. Genotype distribution in patients with and without endometriosis

Genotype	Endometriosis			No endometriosis		
	TT	TG	GG	TT	TG	GG
<i>n</i> (%)	961 (84.2)	177 (15.5)	2 (0.17)	795 (84.8)	133 (14.2)	11 (1.1)
G allele frequency, %			7.90			8.20

analyses. The prevalence of women who were positive for a variant allele was similar ($p = 0.81$; Table 2) in the endometriosis group (15.7%; G allele frequency 7.9%) and in the control group (15.1%; G allele frequency 8.2%). In the previous study variant allele frequency was 18.1% in endometriosis group compared to 7.6% variant allele frequency in the general mixed European population. In order to detect the same difference between the control and endometriosis groups, maintaining a power of 80% with an alpha-I error of 0.05, we would need to test a total of only 316 alleles (158 alleles in each group). In this study, a total of 4,154 alleles were tested, providing sufficient power to detect not only the same difference which we saw in the previous paper but also a difference as small as 2.6%.

Comparison of Patients with and without Endometriosis Carrying Wild Type and Variant KRAS Alleles

The frequency of primary versus secondary infertility in patients with and without endometriosis did not differ depending on the presence of wild type or variant alleles. In cases where the cause of infertility was known (ovulation, implantation, tubal, or genetic), variant allele was

not associated with any individual etiology (Table 3). Patients in all 4 groups had the same BMI and age distribution at the time of surgery. Endometriosis subjects carrying wild type and variant KRAS alleles were not statistically different with respect to their AFS score (30.4 vs. 26.1 in the groups carrying variant vs. wild type alleles, $p = 0.17$) and thus the stage of endometriosis (Table 4). One of the 2 endometriosis patients homozygous for variant allele had an AFS score of 112, while the other did not have an AFS score assigned. Both cases were excluded from the AFS score analysis.

The variant allele did not have any association with BMI, age at surgery, or type of infertility in patients from both groups (with and without endometriosis) when stratification by the presence of endometriosis was removed (Table 5).

Discussion

In our previous study, we found that 31% of patients with moderate to severe endometriosis (undergoing surgery for treatment of pain and failure of medical therapy;

Table 3. Genotype distribution in groups with and without endometriosis stratified by type and causes of infertility

	Endometriosis, <i>n</i> (%)		No endometriosis, <i>n</i> (%)		<i>p</i> value
	WT	KV	WT	KV	
Number	961	179	795	142	0.81
Infertility	905 (94.1)	166 (92.7)	752 (94.5)	137 (96.4)	0.55
Primary	693 (72.1)	129 (72)	476 (59.8)	80 (56.3)	0.59
Secondary	212 (22)	37 (21.5)	276 (34.7)	57 (40.1)	0.42
Ovulation	89 (9.2)	13 (7.2)	152 (19.1)	28 (19.7)	0.4
Implantation	12 (1.2)	3 (1.6)	29 (3.6)	3 (2.1)	0.3
Transport	49 (5)	11 (6.1)	197 (24.5)	25 (14.7)	0.14
Genetics	3 (0.31)	1 (0.5)	8 (1)	1 (7)	0.5
Unexplained	752 (78.2)	138 (77.6)	366 (46)	80 (56.3)	0.25

WT, wild type, TT allele carriers; KV, *KRAS* variant, TG or GG allele carriers.

Table 4. Frequency of wild-type and variant alleles in different stages of endometriosis

	Endometriosis, <i>n</i> (%)		<i>p</i> value
	WT	KV	
Stage			0.49
I	323 (34)	70 (39.1)	
II	205 (21.3)	38 (21.2)	
III	159 (16.5)	29 (16.2)	
IV	262 (27.2)	41 (22.9)	
Unknown	12 (1.2)	2 (1.1)	

WT, wild type; KV, *KRAS* variant.

n = 132; ~90% with stage III-IV endometriosis) were carrying a polymorphism (T-G) in the *let-7* complementary site 6 in the 3'UTR of the *KRAS* gene. We then proposed that this polymorphism may be associated with severe forms of this disease and possibly play a role in its development. However, further large-scale GWA studies failed to demonstrate increased frequency of the variant allele in patients with endometriosis. In this study, we used a much larger patient population from Europe to analyze the frequency of this polymorphism in a case-control study. We used the same direct method of identifying the polymorphism via TaqMan polymerase chain reaction, which was used in our original study. We found the variant allele frequency to be ~8%, which is similar to the previously described frequency of this allele in European population [18]. We did not demonstrate an increased frequency of a variant allele in the patients with endometriosis, nor were we able to identify any association be-

tween the variant allele and other clinical characteristics that were analyzed. We did not see an association between the variant allele and more severe forms of endometriosis. Unfortunately, in this study, we were not able to analyze the association of the *KRAS*-variant with other clinical features of endometriosis such as pain and abnormal uterine bleeding, since this was an infertility cohort and these clinical data had not been documented consistently in the patient files. In our original paper, we did find the polymorphism to be associated with infertility but not with chronic pelvic pain, dysmenorrhea and dyspareunia.

There may be several explanations for the discrepancy of the results of this study and our previous data. The method of identifying the polymorphism was identical in both studies, which minimizes the possibility of procedural differences and errors. All subjects included in this study were diagnosed with infertility, which could have been a confounding factor. All subjects included in our previous study were referred to a university reproductive endocrinology and infertility clinic specializing in endometriosis from community providers, which indicates that the subjects in our former study had undergone and failed multiple therapies and had prior repeated surgeries for endometriosis. In the current study, the subjects were seen for the evaluation of infertility and not selected based on failure of prior therapy. In fact, it is likely than many had no prior therapy. The high incidence of the variant allele in the subjects from our previous study may be a marker of resistance to medical and possibly surgical therapy or nonresponsiveness to infertility treatment rather than an indicator of predisposition to it. Although we did not see enrichment in Stage IV patients in the cur-

Table 5. Comparison of BMI, age at surgery, and types of infertility in patients carrying wild-type or variant alleles

	KV	WT	<i>p</i> value
BMI, mean (SD)	23.5 (4.33)	23.46 (4.34)	0.9
Age at surgery, mean (SD)	32.07 (4.55)	31.65 (4.54)	0.18
Infertility (<i>n</i> = 1,960), <i>n</i> (%)	303 (15.4)	1,657 (84.5)	
Primary (<i>n</i> = 1,378)	209 (15.1)	1,169 (84.9)	0.581
Secondary (<i>n</i> = 582)	94 (16.1)	488 (83.8)	

BMI, body mass index; WT, wild type; KV, *KRAS* variant.

rent study, the high prevalence of *KRAS*-variant patients in our previous cohort of extensively treated patients who had failed standard treatment suggests that *KRAS*-variant patients do have treatment resistant, aggressive endometriosis.

Examples of the existence of subsets of patients who respond differently to standard therapy can be found extensively in cancer. One of the first examples is ER + breast cancer, which comprises up to 2/3 of breast cancer patients. Tamoxifen was one of the first “personalized medicines” applied to better treat these patients. Another example is HER-2/neu oncogene overexpression that is found in approximately 15% of breast cancer patients. While patients with HER-2/neu overexpressing tumors were originally found to have poor outcomes, by using this information to deliver trastuzumab (Herceptin) for the past 10 years, this biomarker is now considered an example of ideal personalized medicine [35]. The *KRAS*-variant is proving to be a strong predictive biomarker in cancer treatment as well, with *KRAS*-variant patients exhibiting cisplatin resistance in ovarian and head and neck cancer [22, 24] and cetuximab sensitivity in colon cancer [25] and head and neck cancer [36]. Thus, the hypothesis that the *KRAS*-variant could be a predictive biomarker of treatment response in endometriosis is not only plausible, but likely. Our previous finding of decreased progesterone receptor expression, increased endometrial cell proliferation rates, and enrichment of *KRAS*-variant patients in high risk treatment resistant endometriosis patients strongly supports the hypothesis that subjects carrying the variant allele may identify a subgroup of endometriosis patients who will have a poor response to standard therapies [27]. This hypothesis deserves further evaluation.

While this study was much better powered and indicates that the *KRAS*-variant rs61764370 is not a marker

of susceptibility to endometriosis in European populations, there are still many questions about the role of the *KRAS*-variant in the pathophysiology of endometriosis. In our original paper, we demonstrated that *KRAS* mRNA expression was increased in eutopic endometrium of subjects with endometriosis compared to endometrium of healthy subjects [27]. *KRAS* overexpression was associated with downregulation of the *let-7* microRNA family in endometrium from subjects with endometriosis, with *let-7b* and *let-7e* being significantly decreased, confirming the known feedback loop between *let-7* and *KRAS*. Similarly, Cho et al. [37] in 2015 demonstrated decreased circulating *let-7* microRNA levels in the serum of subjects with endometriosis. Several other groups also demonstrated elevated *KRAS* mRNA levels in eutopic endometrium of subjects with endometriosis in both human and baboon studies [38–40]. Another group (Laudanski et al. [41]) did not see statistically different expression of *KRAS* mRNA in eutopic endometrium from subjects with and without endometriosis. However, in their study, the levels of *KRAS* mRNA were significantly higher in ovarian endometriosis compared to eutopic endometrium [41]. With more emerging data on *KRAS* and *let-7* expression in subjects with endometriosis (eutopic vs. ectopic endometrium in subjects with endometriosis, eutopic endometrium from subjects with and without endometriosis, serum from subjects with and without endometriosis), it is becoming clear that this interaction plays a meaningful role in the disease; however, the direct mechanism, molecular pathway, effects, and clinical significance of this interaction require more investigation.

In conclusion, a germ-line SNP in the *let-7* microRNA-binding site of the *KRAS* gene was not associated with endometriosis in Caucasian population in a large case-control study; however, it may be a marker of resistance to standard therapies and aggressive disease.

Limitations and Strengths of the Study

Limitations of the study include the retrospective design and that only women with infertility were included. Women with severe pain syndrome, which oftentimes would represent treatment-resistant group, were not included in the study. Also, this was a fairly homogenous

population of Caucasian women of European descent, while in our original study, and there was a substantial number of Hispanic and African-American women. The use of a standardized genotyping as well as laparoscopic confirmation of the presence or absence of the disease in a large population is a significant strength of the study.

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