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KRAS Mutation in Endometriosis-Associated Ovarian Borderline and Malignant Epithelial Tumors

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Abstract

Background: Endometriosis is a common disease among women with the capacity to transform into ovarian neoplasms. KRAS mutation is a keystone in tumor-genesis of many malignant neoplasms.
Objectives: In the current study, we investigated KRAS mutations in endometriosis-associated ovarian borderline and malignant epithelial tumors.
Methods: The specimens of 42 consecutive patients undergoing a surgical procedure whose final diagnosis comprised endometriosis-associated borderline and malignant epithelial ovarian tumors including 12 borderline epithelial tumors and 30 ovarian epithelial carcinomas were histopathologically reviewed. All cases were evaluated regarding the type of tumor, differentiation and simultaneous presence of endometriosis or atypical endometriosis. DNA extraction from the selected paraffin block was done and mutation of codons 12 and 13 was assessed.
Results: Due to the quality of genomic DNA for PCR study was not acceptable in 6 out of 42 cases, among remaining 36 cases, KRAS mutation was observed in 6 cases including 2 cases with mutations in 2nd base of 12th codon (G→T), 3 cases with substitution of G→A in the 2nd base of 12th codon, and one with substitution of G→T in the 1st base of 12th codon.
Conclusions: We evaluated the KRAS mutation in the spectrum of ovarian epithelial tumors associated with endometriosis for treatment approaches including targeted therapies. Our results suggested a possible link between KRAS mutation and endometriosis-associated ovarian borderline and malignant tumors but there was no convincing evidence to prove a definite linkage.

Keywords: Carcinoma, Ovary, Mutation, Endometriosis

1. Background

Endometriosis is a common chronic and painful gynecological disease characterized by the presence of endometrial tissue out of the uterus. The prevalence of endometriosis is not easy to estimate due to some asymptomatic patients (1). Endometriosis leading some symptoms such as dysmenorrhea, infertility, pelvic pain, and reduction in quality of life. There are various hypotheses for endometriosis such as retrograde menstruation, stem cell theory, and coelomic metaplasia (2). Endometriosis which is regarded as a benign and common condition has the can transform and increase the risk of ovarian epithelial tumors, particularly of endometrioid type and clear cell carcinoma; however, the pathogenesis is remained to be elucidated (3, 4). Most ovarian endometrioid carcinomas are associated with endometriosis in the ovary. A transition of atypical endometriosis between endometriotic foci and the invasive tumor is evidence that endometriosis may be a culprit in the pathogenesis of epithelial tumors (5). Ovarian endometriosis may share some features with neoplastic conditions such as monoclonality and loss of heterozygosity (6-8). In addition, the presence of endometriosis in distant areas far from the site of origin is a reminiscence of metastatic behavior of malignancies (9, 10). Many investigators have attempted to determine special molecular alterations in the genome that may lead to the transformation of endometriosis into malignancy. Finding these alterations is helpful to determine whether or not endometriosis is a causative matter in tumorigenesis (11). Due to similarities between endometrioid carcinoma of the uterus and ovary, these two entities may share a common pathogenesis. KRAS mutation has been reported to be the most frequent genetic aberration in uterine endometrioid ade-

nocarcinoma (11).

2. Objectives

In this study, we evaluated the mutation of KRAS in a spectrum of ovarian epithelial tumors associated with endometriosis.

3. Methods

3.1. Sample Collections

In this cross-sectional study all the patients by a final pathology report with the diagnosis of endometriosis-associated borderline and malignant epithelial ovarian tumors undergoing oophorectomy with or without hysterectomy referring to Yas and Imam Khomeini hospital, Tehran University of Medical Sciences, Tehran, Iran during years 2005 until 2015 were evaluated. In this study, only paraffin blocks from archive files and patients' medical records were used. There was no harm to patients' privacy or health in our research. The study has been approved by the committee of ethics of Tehran University of Medical Sciences.

All histological slides were collected and reviewed regarding tumor type; degree of differentiation; and presence of squamous metaplasia (in endometrioid carcinoma), evident endometriosis in available slides, and atypical endometriosis; finally the best paraffin block according to the best slide was designated for polymerase chain reaction (PCR). Then selected paraffin blocks of the patients were gathered and two sections, each measuring five micrometers and one Hematoxylin and Eosin (H&E) slide, as trailing slide were prepared. In order to avoid contamination, we used one blade for each patient's paraffin block.

For atypical endometriosis diagnosis using LaGrenada and Silverberg criteria, as follow:

All foci of endometriosis were examined histopathologically and large nuclei, pale pleomorphic hyperchromasia, nuclear crowding, stratification and tufting, and eosinophilic cytoplasm were recorded. Diagnosis of atypical endometriosis was made in cases with at least three mentioned histomorphological features (12).

3.2. Genomic DNA Extraction and Sequencing

Genomic DNA was extracted using Roche High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the instruction of the manufacturer. Subsequently, 30 to 60 ng of formalin-fixed tumor DNA was amplified in a 20 microliter reaction containing 10 microliters of TaKaRa Ex Taq 2 master mix (Shiga, Japan) and 5 picomole/liter of forward and reverse primers, (forward primer 5'-GGTGAGTTTGTATTAAAAGGTACTGG-3' and reverse primer 5'-GGTCCTGCACCAGTAATATGC-3'). PCR cycling was started with initial denaturation (0.5 min at 94°C), continued by 40 cycles of denaturation (10 s at 95°C), and annealing/extension (30 s at 60°C). In the last step, a final extension (10 min at 72°C) was added. All the samples were kept at 4°C until they were sent for sequencing Macrogen (Seoul, Korea) for direct Sanger sequencing using reverse primer. The sequencing result was analyzed using Chroma software version 2.6. The target sequence was used as an amplification control. Moreover, those samples that showed a mutation in either codon 12 or codon 13 were re-amplified and re-sequenced using forward primer for confirmation.

3.3. Statistical Analysis

Finally, in this descriptive study, all the gathered data entered Microsoft Excel software. Results of qualitative variables were reported as frequency, and percent and results of quantitative variables were calculated and reported as mean and standard deviation.

4. Results

We identified a total number of 42 patients with endometriosis-associated borderline and malignant epithelial ovarian tumors including 12 patients with borderline epithelial tumor and 30 patients with ovarian epithelial carcinoma.

The mean age $\pm$ SD was 43 $\pm$ 11.3 years (range, 22 - 67), definite pathological diagnosis, and frequency of each type of epithelial tumor and association with endometriosis are shown in Tables 1 and 2, respectively. The mean diameter of the tumor was 11.9 $\pm$ 6 cm (range, 4 - 30 cm). The mean number of paraffin blocks per case was 16.2 $\pm$ 7.1 (range, 6 - 36). One paraffin block was prepared for each centimeter of tumor except in four cases (including two endometrioid carcinomas, one clear cell carcinoma on one side and seromucinous borderline tumor, and one mucinous borderline tumor on the other side.)

4.1. Genomic DNA Evaluation

Due to inappropriate fixation technique and DNA destruction, the quality of genomic DNA for PCR study was not acceptable in 6 out of 42 (14.3%) cases. Among the remaining 36 cases, KRAS mutation was observed in 6 (16.7%) cases. In two cases mutations in the 2nd base of the 12th codon were (G→T) detected, which histological diagnosis of tumor type was seromucinous borderline tumor and clear cell carcinoma. In three cases substitution of G→A in

Table 1. Frequency of Epithelial Ovarian Tumors with and Without Endometriosis

Final Histological Diagnosis	Number of Cases
Clear cell carcinoma	11
Endometrioid carcinoma	18
Non-invasive papillary tumor arising from endometrioid cyst	1
Bilateral serous borderline and mucinous borderline tumor	1
Endometrioid borderline tumor	1
Seromucinous borderline tumor	3
Mucinous borderline tumor	4
Serous borderline tumor	3
Total	42

the 2nd base of the 12th codon was seen. Histological diagnosis tumor types in these three cases were endometrioid carcinoma, serous borderline tumor, and bilateral seromucinous borderline tumor. In one case with histological diagnosis of clear cell carcinoma on one side and seromucinous borderline tumor, on the other side, substitution of G→T in the 1st base of the 12th codon was seen. The characteristics of tumors with KRAS mutations are shown in detail in Table 3.

5. Discussion

Mutations in the KRAS gene occur in approximately 20% of all human tumors. The occurrence of irreversible alteration of the 12th codon of KRAS gene has been found to be early during the development of lung adenocarcinoma (13). Among gynecological pathologies, KRAS mutations were identified in endometrial hyperplasia with a similar prevalence to that found detected in endometrial carcinoma, therefore, it is postulated that KRAS may play a role in the early step of carcinogenesis (14-16).

Similarly, numerous studies have evaluated the association of ovarian epithelial tumors with KRAS mutations (13). Higher rate of KRAS mutation has been found in serous borderline tumors (27 - 36%) than in high-grade serous carcinoma (0 - 12%). Other studies showed that low-grade and high-grade serous carcinomas have different pathogenesis. The hypothesis of adenoma-carcinoma sequence with KRAS or BRAF mutation has been suggested for low-grade serous carcinomas which means progression from typical to micropapillary borderline tumors to low-grade serous carcinoma (3, 4, 13-16). In our study, KRAS mutation was also detected in low-grade epithelial ovarian tumors FIGO stage I.

The association of endometriosis with ovarian epithelial tumors is well-established in the literature and since Sampson first described this association in 1925, many subsequent studies including molecular studies were performed to discover the molecular pathway of endometriosis-associated tumor genesis (17). Among all ovarian epithelial tumors, clear cell carcinoma, endometrioid carcinoma, and seromucinous borderline tumor to a lesser extent, are associated with endometriosis (18). In some of the mentioned tumors, there is a continuum of the sequential morphological spectrum from endometriotic focus to tumor. In the current study, among 36 epithelial tumors of different categories, KRAS mutation was found in 6 patients includingendometrioid carcinoma, clear cell carcinoma, and seromucinous borderline tumor, while the association of KRAS mutation and tumor type was not statistically significant in our study but this mutation occurred in three tumor types which are reported to be highly associated with endometriosis.

KRAS mutation has been reported to occur in approximately 10% of endometrial carcinomas, although the incidence of this mutation in various studies ranged from 0% up to 36% (19-21). These significant variations in the reported frequency of KRAS mutation in endometriosis-associated ovarian tumors can be due to the small sample size and different histologic grades of the tumors (22-24). Stwart et al. evaluated the molecular alterations in KRAS gene in endometrioid adenocarcinoma (FIGO grade 1 and 2) including 42 patients with endometriosis-associated endometrioid adenocarcinoma and 29 patients with independent endometrioid adenocarcinoma. KRAS mutation was detected in 29% of the former while it was identified in 3% of the latter. The study suggested KRAS mutations can play a role in the pathogenesis of the former tumors (21). In the present study, 18 out of a total of 42 cases were diagnosed as endometrioid carcinoma with endometriosis. Nine cases were well-differentiated and eleven cases had accompanying squamous metaplasia. KRAS mutation was found in 2 (11%) cases of well-differentiated endometrioid carcinoma. In both of the mentioned cases, KRAS mutation was detected in the 2nd base of 12th codon G→A. One of them had also atypical endometriosis. Both of them had squamous metaplasia.

Although the pathogenesis of endometriosis has not been fully elucidated, one of the current theories is retrograde transtubal spread, implantation and subsequent proliferation of endometrial tissue within ovaries and other peritoneal sites. Thus, endometriosis originates from eutopic endometrium. The similar spectrum of genetic alteration in endometriosis-associated carcinomas and their uterine counterparts may support this theory of the origination of endometriosis (25). The presence of the same codon-KRAS mutation in two cases of endometriosis associated well-differentiated endometrioid

Table 2. Detailed Categorization of Epithelial Ovarian Tumors with and Without Endometriosis

Tumor Diagnosis	Endometriosis	Presence of Atypical Endometriosis
Endometrioid carcinoma	12	2
Bilateral endometrioid carcinoma	1	1
unilateral endometrioid carcinoma and endometrioid borderline tumor	1	-
Endometrioid carcinoma in one side and serous borderline tumor in the other side	1	-
Unilateral endometrioid and serous carcinoma	1	-
Unilateral endometrioid and clear cell carcinoma	3	-
Clear cell carcinoma	7	3
Bilateral clear cell carcinoma	1	-
Clear cell carcinoma in one side and seromucinous borderline tumor, in the other side	1	1
Clear cell carcinoma in one side and endometrioid borderline tumor in the other side	1	1
Bilateral serous borderline tumor	1	-
Mucinous borderline tumor	4	-
Seromucinous borderline tumor	3	1
Endometrioid borderline tumor	1	-
Serous borderline tumor	2	2
Bilateral serous borderline and mucinous borderline tumor	1	-
Non-invasive papillary tumor arising from endometriotic cyst	1	1
Total	42	12

Table 3. Frequency of Squamous Metaplasia, Degree of Differentiation and Tumor Stage in Patients with KRAS Mutation

Tumor Type	Age	Endometriosis	Atypical Endometriosis	Squamous Metaplasia	Degree of Differentiation	FIGO Stage	KRAS Mutation
Endometrioid carcinoma	58	Yes	Yes	Yes	Well differentiated	IA	2nd base of 12th codon G>A
Endometrioid carcinoma and serous borderline tumor	38	Yes	No	Yes	Well differentiated	IC2	2nd base of 12th codon G>A
Clear cell carcinoma and seromucinous borderline tumor	46	Yes	Yes			IA	1st base of 12th codon G>T
Clear cell carcinoma	43	Yes	No			IC2	2nd base of 12th codon G>T
Seromucinous borderline tumor	25	Yes	No			IA	2nd base of 12th codon G>T
Bilateral seromucinous borderline tumor	45	Yes	Yes			IB	2nd base of 12th codon G>A

carcinoma, FIGO stage 1 with squamous metaplasia supports the hypothesis that endometriosis-associated well-differentiated endomerioid carcinoma progress through certain molecular pathways which may have implications for future treatment approaches including targeted therapies of patients.

In a study by Otsuka et al. KRAS mutation was evaluated in 37 patients with clear cell carcinoma and mutation of KRAS was found in six cases. Three out of six specimens contained adjacent typical or atypical endometriosis. Molecular studies using direct sequencing of the 12th codon in tumoral cells and adjacent atypical endometriotic areas were performed separately. KRAS mutation was detected in tumoral areas but it was negative in adjacent foci of endometriosis. This observation postulated that ovarian clear cell carcinoma may arise from KRAS mutation in the

endometriotic part (26). In the present study, KRAS mutation was identified in only 2 out of 11(18%) cases of ovarian clear cell carcinomas. Detected mutations were in the 1st base of the 12th codon G→T the 2nd base of the 12th codon G→T. Cuatrecasas et al. reported that the frequency of codon 12/13 KRAS gene mutations was 31.3% (5/16) in clear cell carcinomas and 36.4% (12/33) in endometrioid carcinomas, and Okuda et al. found KRAS codon 12/13 mutations in 16.2% of clear cell carcinomas and 3.7% of endometrioid carcinomas (22, 27, 28). In the present study, we detected KRAS codon 12/13 mutations in 18% of clear cell carcinomas and 11% of endometrioid carcinomas with frequencies closer to Okuda study. One possible explanation for discrepancies between various studies is that the prevalence of KRAS mutations differs according to an ethnic group or geographic region.

In 1993, Mok et al. performed a study to detect KRAS mutations in endometriosis-independent epithelial ovarian neoplasms. KRAS mutation was found in 21 out of 44 including 19 mucinous and 25 serous borderline tumor cases of this study. All of the cases had mutations in the 12th codon except one mucinous borderline tumor with the 13th codon mutation. In Mok series, KRAS mutation was seen in 12 out of 19 mucinous borderline tumors, including 9 tumors with mutation of 2nd base of 12 codon G→T, 2 cases of 2nd base of 12th codon G→A, and one case with 2nd base G→A substitution (24).

In our study, KRAS mutation of the 2nd base of 12th codon G→T and 2nd base of 12th codon G→A was also found in two cases of seromucinous borderline tumor, and bilateral seromucinous borderline tumor, respectively. These findings are in concurred with Mok series; also, in our study, all the cases with KRAS mutation were categorized as stage I. KRAS mutation is common in mucinous ovarian tumors and in seromucinous borderline tumors. This genetic alteration has been identified in histologically benign epithelium adjacent to borderline serous or mucinous elements suggesting that these mutations occur early during neoplastic development (24, 26).

One of the advantages of the current study is the presence of several mixed ovarian epithelial tumors and detailed sequencing of the 12th and 13th KRAS codon; however, there were some limitations regarding method of sequencing. Sanger sequencing was the method used to detect mutations of KRAS gene. This method is unable to detect mutations that are present in less than 20% of tumor cells (29, 30). For detecting more mutated cells, new generation sequencing is helpful. In addition, in our study mutation of KRAS is assessed in the 12th and 13th codons. Other codons such as 59th, 61st, and 146th may also have mutations that we did not evaluate in this study.

In conclusion, this is the first study, which evaluated the association between endometriosis-associated mixed ovarian epithelial tumors and KRAS mutation. Our results did not provide convincing evidence to prove a definite linkage. However, only a few cases were included in this report, and therefore further research will be needed to define the role of KRAS in the development of endometriosis-associated ovarian epithelial tumors.

Footnotes

Authors' Contribution: The authors contributed extensively to the work presented in this paper. Dr. Soheila Sarmadi and Marzieh Fakhari helped in gathering data. Dr Maniya Mozafari prepared and edited the article. Dr Reza Shahsiah, Dr Narges Izadi-Mood and Dr. Rosa Miri helped in reviewing pathological reports and molecular studies.

Conflict of Interests: There are no conflict of interests.

Data Reproducibility: The data presented in this study are openly available in one of the repositories or will be available on request from the corresponding author by this journal representative at any time during submission or after publication. Otherwise, all consequences of possible withdrawal or future retraction will be with the corresponding author.

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Informed Consent: No human subject was directly involved in this study. We used only archived paraffin blocks and medical records.

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