

Mutations in the *KRAS* gene in ovarian tumors

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Abstract: *RAS* genes are the most frequently mutated oncogenes detected in human cancer. In this study we analyzed the presence of mutations at codon 12 of the *KRAS* gene in 78 women with ovarian tumor, including 64 invasive ovarian cancers and 14 borderline ovarian tumors, using an RFLP-PCR technique and we evaluated whether such alterations were associated with the selected clinicopathological parameters of the patients. *KRAS* codon 12 gene mutations were found in 6,2% of ovarian cancer tissue and in 14,3% of the borderline ovarian tumor. *KRAS* mutations were found with a significantly higher frequency in mucinous and borderline tumors compared to serous tumors ($p < 0,01$). Mutation frequency was correlated with the histological type of tumor, but not with stage, grade or patients age.

Key words: ovarian tumor, *KRAS*, point mutation, molecular detection

Introduction

Ovarian cancer is the sixth most frequent female cancer type and the sixth most frequent cause of death from cancer among women in Poland. Approximately 2700 new cases appear every year, constituting an age-standardized incidence rate of 14,4 per 100 000, which is one of the highest incidence rates observed worldwide [1].

Most human malignancies are the end result of an accumulation of mutations within tumor-suppressor genes and oncogenes as well as of the dysregulation of specific genes resulting in the antiapoptotic proteins eliminations [2]. Molecular studies have identified several genetic alterations such as p53, *KRAS*, c-erbB-2, *PTEN*, *HER-2/neu* and *BRCA1* mutations in ovarian tumors [3,4].

The *RAS* family of oncogenes is constituted of three principal members -*KRAS*, *HRAS* and *NRAS* – all of which have been implicated in the development of human malignancies. The *KRAS* oncogene resides on chromosome 12p12 and encodes a 21-kD protein

(p21*RAS*) involved in the MAP-kinase signal transduction pathway, modulating cellular proliferation and differentiation. Mutations of the *KRAS* oncogene result in constitutive activation of this signal transduction pathway and consequently unregulated proliferation and impaired differentiation [5,6].

Activation of *RAS* oncogenes also occurs in ovarian tumors. Some studies have shown that *KRAS* mutations are more frequent in mucinous than in nonmucinous neoplasm [7-9], whereas other studies have not revealed correlation with histological type [10]. All the reported studies are based on a relatively small number of patients and therefore, the results remain a subject of debate.

In this study, we analyzed the presence of mutations at codon 12 of the *KRAS* gene in 78 ovarian tumors by using a restriction fragment length polymorphism-polymerase chain reaction technique (RFLP-PCR) and we evaluated whether such alterations correlated with the selected clinicopathological parameters of the patients.

Materials and methods

In this study, we analyzed the presence of mutations at codon 12 of the *KRAS* gene in 78 women with ovarian tumor, including 64 invasive ovarian cancers and 14 borderline ovarian tumors. All tumors were staged according to the criteria of the International

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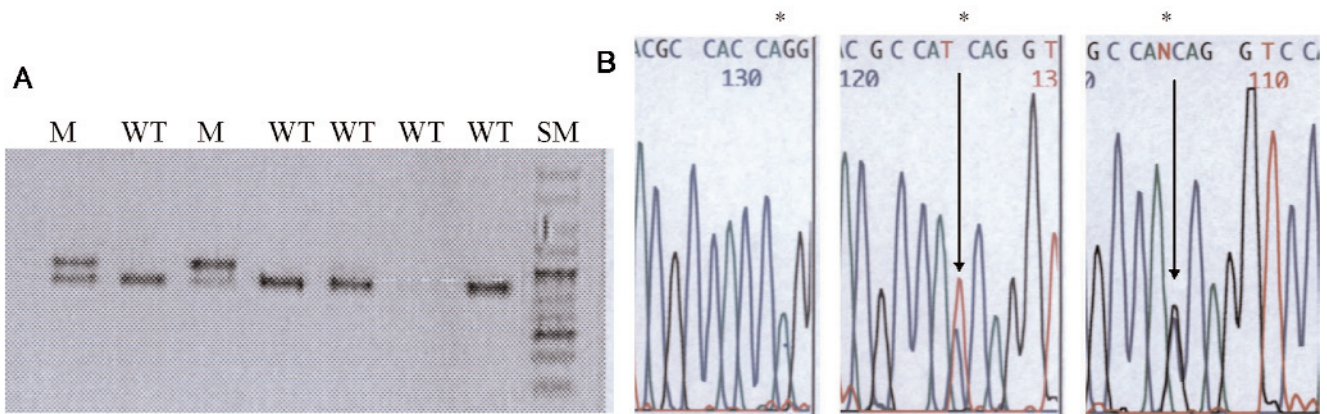


Fig. 1. Examples of mutation analysis of *KRAS* gene. (A) *KRAS* codon 12 restriction fragment length polymorphism analysis. (B) A sequencing electropherograms with *KRAS* codon 12 mutants. (←) mutation in *KRAS* gene, (*) a mismatch base in artificial forward primer, SM, size marker; WT, wild type, M, mutant.

Federation of Gynecology and Obstetrics (FIGO). The median age of the patients was 58 (range, 34–75) years. The protocol was previously approved by the Bioethical Committee of the Medical University of Białystok (R-I-003/206/2004).

Serial paraffin sections were stained with H&E for light microscopic study. Clinicopathological information was obtained from medical charts. Histopathological examination was performed according to the WHO classification. Tissues were immediately frozen in liquid nitrogen after operation and stored at -80°C until use. We microscopically confirmed that the tumor specimens consisted mainly of carcinoma tissue (80%). DNA extraction was performed with a "GeneElute Mammalian Genomic DNA Miniprep Kit" (Sigma), according to the manufacturer's instructions. Before DNA extraction, tumor samples (30–50 mg) were minced by the use of a sterile scalpel and then digested for overnight at 37°C in 180 μl of tissue lysis buffer from the kit, containing 20 μl of proteinase K solution (10 mg/ml). Isolated DNA was stored at -20°C before further assays.

The detection of *KRAS* mutations at codon 12 was performed by PCR-RFLP method and the results of the detection were verified by direct sequencing of PCR products. DNA amplification was performed in 20 μl reaction mixture containing 10–100 ng of genomic DNA isolated from tissue, 1.5 mM of MgCl_2 , 0.2 mM of dNTPs (Sigma), 0.2 M of each of the primers K1 and DD5P and 1.0 U of *Taq* DNA polymerase (Sigma) in $1\times$ PCR buffer supplied by the polymerase manufacturer. The K1 upstream primer (5'-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT -3') was immediately upstream of *K-RAS* codon 12 and introduced a G to C substitution at the first position of the codon 11 of *K-RAS* in order to create a *Bst*OI restriction site (5'-CCT/AGG-3') within the above amplified fragment which overlapped the first two nucleotides of codon 12 and was lost when codon 12 mutations took place. The downstream primer DD5P was as follows: 5'-TCA TGA AAA TGG TCA GAG AA-3'.

After the initial DNA denaturation at 95°C for 3 min, PCR was carried out for 40 cycles (94°C for 15 s, 56°C for 15 s, 72°C for 15 s) followed by terminal extension of PCR products at 72°C for 7 minutes. PCR products were then digested with a restriction endonuclease *Bst*OI. For this purpose, five-microliter aliquots of the post-PCR reaction mixture were digested with 10 U of the restriction endonuclease *Bst*OI (PROMEGA) in the appropriate reaction buffer (supplied by the enzyme manufacturer) in the final volume of 10 μl at 60°C for 3 h. The additional aliquot of 5 U of the enzyme was added to the reaction mixture after the first hour of the digestion. The enzyme recognized the sequence 5'-CCTGG-3', which was present in codon 12 *KRAS* wild type PCR products, but was absent from the mutant ones. As a result, only the wild type

molecules were digested into two fragments – 160 bp and 29 bp long. The digestion products were then electrophoresed on a 6% native polyacrylamide gel, stained with ethidium bromide and photographed on a ultraviolet light transilluminator with the use of UVI-KS 400i/Image PC system. The non-restricted PCR products were 189 bp long, whereas the wild type codon 12 products, being restricted inside codon 12 sequence, were 160 bp long.

All mutations were then confirmed by direct sequencing of the PCR products. For this purpose, antisense strain of PCR products were sequenced with the antisense primer DD5P, an ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing Ready Reaction (Applied Biosystems) and an automatic ABI PRISM 377 DNA sequencer (Applied Biosystems). A wild-type control DNA sample (without *KRAS* codon 12 mutation) and a known mutation sample were included in all the experiments. All experiments were duplicated precisely. For statistical analysis, the χ^2 test was performed; $p < 0.01$ was considered significant.

Results

The age of the patients ranged from 34 to 75 years (mean, 58). *KRAS* gene mutations at codon 12 in tumor tissue were detected in 4 of 64 (6.2%) cases with primary invasive ovarian carcinomas and in 2 of 14 (14.3%) cases with a borderline ovarian tumor (Fig. 1).

We detected *KRAS* gene mutation in 1 of 36 serous adenocarcinomas (2.7%), 3 of 13 mucinous adenocarcinomas (23%) and 2 of 14 borderline ovarian tumors (14.3%). No mutations were detected in undifferentiated carcinomas, papillary adenocarcinomas, endometrioid adenocarcinomas, nonepithelial carcinomas and in clear cell neoplasms (Table 1, 2).

Overall, a *KRAS* codon 12 point mutation was detected in approximately 10% (8/78) of the examined tumors and did not correlate with the malignant potentials (e.g. stage and grade) and patients' age (Table 1). There was a tendency towards a higher incidence of *KRAS* mutations in the mucinous tumors – 23% (3/13) than in the borderline and serous tumors – 14.3% (2/14) and 2.7% (1/36), respectively. These statistical differences are significant ($p < 0.01$).

Table 1. Distribution of *KRAS* mutations according to clinicopathological characteristics of the examined ovarian lesions.

Parameters	Number of cases	Cases with <i>KRAS</i> mutations (%)	p value
Overall	78	8 (10.3)	
Age at diagnosis (year)			NS
<50	9	1 (11)	
50-60	31	3 (9.7)	
>60	38	4 (10.5)	
Histology			p<0.01
Serous adenocarcinoma	36	1 (2.7)	
Mucinous adenocarcinoma	13	3 (23)	
Undifferentiated carcinoma	2	0 (0)	
Papillary adenocarcinoma	3	0 (0)	
Endometrioid adenocarcinoma	5	0 (0)	
Nonepithelial carcinoma	3	0 (0)	
Clear cell neoplasm	2	0 (0)	
Borderline ovarian tumor	14	2 (14.3)	
FIGO stage			NS
I	0	0 (0)	
II	38	4 (10.5)	
III	33	4 (12.1)	
IV	7	0 (0)	
Cellular grade			NS
G ₁	29	3 (10.3)	
G ₂	37	4 (10.8)	
G ₃	12	1 (8.3)	

Discussion

Epithelial ovarian tumors are a complex clinical, diagnostic and therapeutic challenge because of the difficulty of early detection, lack of known precursor lesions and high mortality rates. Advanced-stage disease display aggressive chemoresistant behavior with patients demonstrating a shorter median survival rate [11,12].

Extensive studies have been performed on human samples assessing the presence of activated forms of *KRAS* in numerous malignant neoplasms, e.g.: pancreas (60%), biliary tract (32%), large intestine (32%), small intestine (20%), gastrointestinal tract (site indeterminate) (19%), thymus (15%) and endometrium (14%) [13].

Table 2. *KRAS* gene mutations in the examined ovarian lesions.

Histologic type	<i>KRAS</i> gene mutations (%)
Serous adenocarcinoma	1/36 (2.7)
Mucinous adenocarcinoma	3/13 (23)
Undifferentiated carcinoma	0/2 (0)
Papillary adenocarcinoma	0/3 (0)
Endometrioid adenocarcinoma	0/5 (0)
Nonepithelial carcinoma	0/3 (0)
Clear cell neoplasm	0/2 (0)
Borderline ovarian tumor	2/14 (14.3)

In epithelial ovarian cancers, the incidence of *KRAS* point mutations is between 15% and 39% [13,14]. In our study *KRAS* mutation frequencies seem to be highly related to tumor histology. There was a tendency towards a higher incidence of *KRAS* mutation in the mucinous tumors (23%) than in the borderline and serous tumors (14.3% and 2.7%), respectively. In general, *KRAS* mutations occur more frequently in mucinous tumors, including borderline malignancies, than in nonmucinous tumors such as serous carcinomas. Thus, point mutations in the *KRAS* gene seem to be more commonly associated with mucinous carcinomas [15].

Our earlier study showed that mucinous borderline tumors are clearly distinct from their serous counterparts [16,17]. In this study we found that more mucinous ovarian tumors have *KRAS* mutations than borderline tumors and other invasive cancers. Thus, both mucinous ovarian cancer and borderline ovarian tumor are basically different from other ovarian cancers since the former are initiated by specific mutations (e.g. *KRAS*) while the latter are initiated by different mechanisms [18].

A few previous studies have determined the prognostic role of *KRAS* alteration in ovarian cancer [19]. Several studies found no correlation between *KRAS* gene mutations and survival [10,20], while very few concluded that there is a significant negative relationship using multivariate analysis [21].

We found 3/13 of the investigated mucinous ovarian cancer tissues (23%) to have a mutation in *KRAS* codon 12, which was the highest frequency detected in a subgroup of patients in this study, and significantly higher than the frequency of 1/36 for serous adenocarcinomas (2.7%). All the two borderline ovarian tumors (2/14) found to have a mutation in *KRAS* codon 12 were mucinous (14.3%). Thus, *KRAS* mutations frequency in our study was correlated with the histological type of tumor but not with other clinicopathological parameters such as grade, stage or patients age.

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