

Mutations of the *KRAS* oncogene in endometrial hyperplasia and carcinoma

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Abstract: The aim of this study was to examine the prevalence and clinicopathological significance of *KRAS* point mutation in endometrial hyperplasia and carcinoma. We analysed *KRAS* in 11 cases of complex atypical hyperplasia and in 49 endometrial carcinomas using polymerase chain reaction associated with restriction fragment length polymorphism (PCR-RFPL). Point mutations at codon 12 of *KRAS* oncogene were identified in 7 of 49 (14.3%) tumor specimens and in 2 of 11 (18.2%) hyperplasias. No correlation was found between *KRAS* gene mutation and age at onset, histology, grade of differentiation and clinical stage. We conclude that *KRAS* mutation is a relatively common event in endometrial carcinogenesis, but with no prognostic value.

Key words: endometrial carcinoma, endometrial hyperplasia, *KRAS*, point mutation, molecular detection

Introduction

Endometrial cancer (EC) develops in about 142000 women worldwide annually being the reason of death in about 42000 women. The most common lesions are typically hormone sensitive and low stage endometrioid cancer which have an excellent prognosis (EC type 1). In contrast, EC type 2 (serous carcinoma) is high grade with a tendency to recur, even at an early stage [1].

The *RAS* family G proteins (*N-*, *H-*, and *KRAS*) are thought to play a critical role in the regulation of cellular proliferation. On the other hand evaluation of molecular alterations involved in the regulation of tumor growth, cell death and markers of cancer progression have provided the fundamental basis for the development of molecular targeted therapies [2].

Mutations of the *KRAS* gene have been implicated in the development of numerous endometrial malignancies [3-7]. The mutation in codon 12 of *KRAS* has been found in 10-30% of endometrial carcinomas. Some studies have indicated that mutations in codon

12 of *KRAS* are an independent, unfavourable prognostic factor [8-10] while others have not found this correlation [11-15].

A higher prevalence of *KRAS* mutations in endometrial carcinomas in Japan (30%) compared with other countries (15%) has been indicated [11,12,14]. In addition, *KRAS* mutations have been identified in endometrial hyperplasias (EH) and more frequently in complex atypical hyperplasias (CAH), which suggests the *KRAS* mutations may be a relatively early event in endometrial carcinogenesis.

In connection with these data, we investigated mutation status of codon 12 of *KRAS* in surgical specimens of endometrial carcinoma and endometrial hyperplasia using a polymerase chain reaction associated with restriction fragment length polymorphism (PCR-RFPL).

Materials and methods

Tissue samples. 49 patients with histologically confirmed diagnosis of endometrial carcinoma were enrolled into the study (mean age \pm SD – 62.25 \pm 3.75 years). All tumors were staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). Finally, an additional diagnosed set of 11 CAH was included. The initial manifestation of all cases of hyperplasia was an abnormal vaginal bleeding and the age range of patients was 40-52 years. Serial paraffin sections

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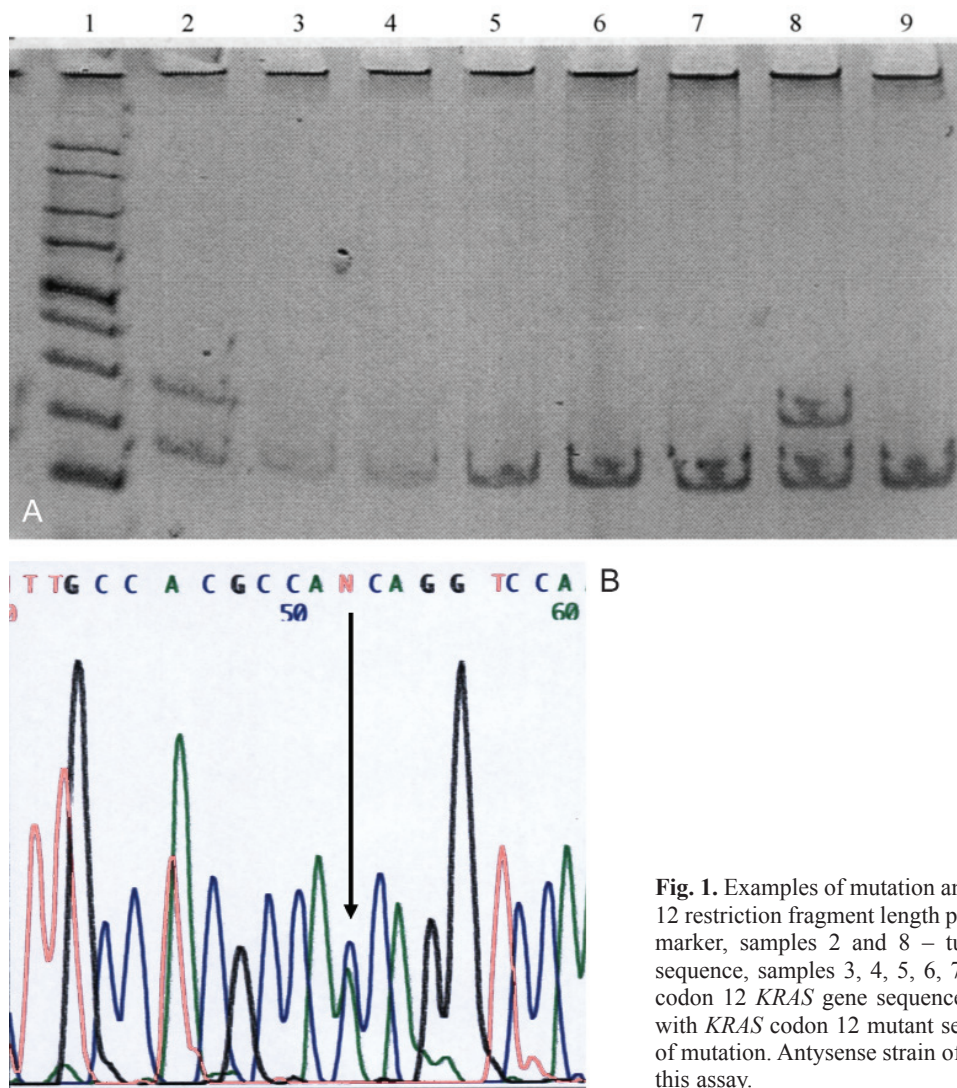


Fig. 1. Examples of mutation analysis of *KRAS* gene. (A) *KRAS* codon 12 restriction fragment length polymorphism analysis. Sample 1 – size marker, samples 2 and 8 – tumors with mutated codon 12 *KRAS* sequence, samples 3, 4, 5, 6, 7, 9 – tumors with normal (wild type) codon 12 *KRAS* gene sequence. (B) A sequencing electropherogram with *KRAS* codon 12 mutant sequence. The letter N indicates the site of mutation. Antysense strain of the *KRAS* gene has been sequenced in this assay.

were stained with H+E for light microscopic study. Clinico-pathological information was obtained from medical charts and histopathological examination was performed according to the WHO classification. None of the patients had received radiation or hormonal therapy prior to surgery. Only 3 patients were premenopausal and the remaining 46 were postmenopausal.

RFLP. 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 μ l reaction mixture containing 10 – 100 ng of genomic DNA isolated from tissue, 1.5 mM of MgCl₂, 0.2 μ M of dNTPs (Sigma), 0.2 M of each of the primers K1 and DD5P and 1.0 U of *Taq* DNA polymerase (Sigma) in 1x 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 *KRAS* codon 12 and introduced a G to C substitution at the first position of the codon 11 of *KRAS* 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'.

Sequencing. 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 μ l 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

Table 1. Distribution of *KRAS* mutations according to clinicopathological characteristics of the examined tumor specimens.

Parameters	Number of cases	Cases with <i>KRAS</i> mutations (%)	p-value
Overall	49	7 (14.3)	
Age at diagnosis (year)			NS
<53	3	0 (0)	
54-59	7	2 (28.6)	
>60	39	5 (12.8)	
Histology			NS
Endometrioid	39	7 (17.9)	
Adenoacanthoma	7	0 (0)	
Clear Cell Carcinoma	2	0 (0)	
Papillary Serous Carcinoma	1	0 (0)	
FIGO stage			NS
I	17	3 (17.6)	
II	26	3 (11.5)	
III	6	1(16.7)	
Cellular grade			NS
G ₁	28	5 (17.9)	
G ₂	14	1 (7.1)	
G ₃	7	1 (14.3)	

v.3.1 Cycle Sequencing Ready Reaction kit (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.

Ethical issues. The protocol was previously approved by the Bioethical Committee of the Medical University of Białystok (R-I-003/177/2004).

Statistical analysis. For statistical analysis, the χ^2 test was performed; $p < 0.05$ was considered significant.

Results

The stage of the 49 patients was: Ia – 3 cases, Ib – 10, Ic – 4, IIa – 20, IIb – 6, IIIa – 4, IIIb – 1 and IIIc – 1 cases. Histologically, 39 of the 49 patients had endometrioid – type carcinoma, 7 had adenocarcinoma with squamous differentiation, 1 had papillary serous carcinoma and 2 had clear cell carcinoma. 28 were well-differentiated (G₁), 14 were moderately differentiated (G₂) and 7 were poorly differentiated (G₃).

KRAS mutation was detected in 7/39 (14.3%) of tumor specimens and in 2/11 (18.2%) of CAH (Fig. 1). The results are summarized in Tables 1 and 2.

Discussion

Although EC is the most common gynecologic cancer in the developed world, the details of its carcinogenesis are still not well known. In the majority of cases, the neoplasm is histologically diagnosed as the endometrioid type (type 1) and its stage at the time of diagnosis is determined as I (FIGO). Some histological properties of EC have a prognostic value as they are associated with the risk of metastases, recurrence and the length of survival. Such prognostic factors include the histological type, grade, depth of myometrium infiltration and lymph-vascular space involvement. Recently, a number of steps in sporadic endometrial carcinogenesis have been identified. The most important basis of understanding molecular events in EC is that this is a hormone-dependent tumor. Additional molecular abnormalities include overexpression of p53 and *KRAS* point mutations [16,17].

RAS oncogenes are the proven proto-oncogenes in human activated by point mutation. The three *RAS* oncogenes, *HRAS*, *KRAS*, and *NRAS*, share a common amino acid sequence, with a subtle difference in C-terminal part of the polypeptides. The point mutation in codon 12 of *RAS* oncogene changes glycine to other

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

Histologic type	<i>KRAS</i> gene mutations no. (%)
Complex atypical hyperplasia	2/11 (18.2)
Endometrioid carcinoma	7/39 (17.9)
Adenoacanthoma	0/7 (0)
Clear Cell Carcinoma	0/2 (0)
Papillary Serous Carcinoma	0/1 (0)

amino acids except proline, and this transformation results in carcinogenesis.

KRAS point mutations have been implicated in the development of atypical endometrial hyperplasia and EC. These findings suggest that *KRAS* mutations may be an early oncogenic event in endometrial carcinogenesis [18]. In the case of EC, molecular details are beginning to emerge, which may eventually help advance our understanding of the complex histopathology of this disease. The estrogen-related type 1 EC account for up to 80% of the EC cases and appear to arise via a progression pathway. Frequently, the advanced carcinomas are temporally and spatially associated with complex atypical hyperplasia, which constitutes a range of heterogeneous precancerous lesions. Atypical hyperplasia, the most advanced component of this histopathologic spectrum, is considered to be the direct precursor lesion of type 1 EC. Subsets of type 1 EC were found to contain *KRAS* mutations (15-20%). Both of these abnormalities were also detected in CAH [19]. Fujimoto et al. reported a 22% *KRAS* point mutation rate, and this abnormality was associated with high rate in lymph node metastasis [20]. Mizuuchi et al. reported *KRAS* point mutation as a poor prognostic parameter in EC [9]. Relatively high incidences of *KRAS* mutations have been reported for EC, especially for type 1 carcinoma, suggesting that mutant *KRAS* is involved in the development and/or progression of these tumors [8-10]. In the present study, no correlation was found between *KRAS* gene mutation and age at onset, histological subtype, grade of differentiation and clinical stage. We conclude that point mutations at codon 12 of *KRAS* oncogene are a relatively common event in endometrial carcinogenesis, but their prognostic value is limited.

Acknowledgements: This study was supported by grant 2P05E00726 of Ministry of Science and Higher Education of Poland.

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Submitted: 20 July, 2008

Accepted after reviews: 4 September, 2008