K-*ras* gene point mutations and p21^{ras} immunostaining in human ovarian tumors

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Summary

It is well recognized that genetic alterations within oncogenes, tumor suppressor genes, DNA mismatch repair and excision repair genes contribute to tumorigenesis within the human ovary. This study was undertaken to screen for the existence of K-*ras* gene point mutations in paraffin-embedded slides randomly selected from benign and malignant ovarian tumors applying the PCR-RFLP technique. Expression of p21^{ras} was also assessed in 30 primary ovarian adenocarcinomas immunohistochemically. K-*ras* codon 12 point mutations occurred in nine of 40 (22.5%) cases. They were not identified in two benign mucinous cystadenomas, but in one out of two (50%) mucinous tumors of LMP (low malignant potential), in five out of 30 (17%) ovarian adenocarcinomas, and in one case of adenocarcinoma metastatic to the ovary. K-*ras* activation was also detected in one out of four (25%) sex cord-stromal cell tumors (folliculoma), and in one dysgerminoma. None of these tumors exhibited K-*ras* codon 13 point mutations. Gene alterations were more frequently found in mucinous than in non-mucinous (30% vs 10%) tumors, although the difference did not reach significance ($p > 0.05$). The frequency of K-*ras* point mutations was correlated neither with clinical nor with pathological variables of cancer. Cytoplasmic p21^{ras} was expressed in all adenocarcinomas negative for K-*ras* point mutations, whereas one of five (20%) K-*ras*-positive tumors exhibited lack of immunoreactivity. In conclusion, these findings confirm the role of K-*ras* activation in mucinous ovarian tumors. p21^{ras} expression is not necessarily associated with K-*ras* gene alterations in human ovarian adenocarcinomas.

Key words: K-*ras;* Ovarian cancer; PCR; RFLP.

Introduction

Accumulation of various genetic derailments, including activation of oncogenes and growth factors, as well as inactivation of tumor suppressor genes, DNA mismatch repair and excision repair genes, appears to be implicated in the development of ovarian cancer [1, 2]. One of the well-studied oncogenes, the full-length sequence of which was cloned nearly 20 years ago, was the K-*ras* [3, 4]. This oncogene, spanned at chromosome 12p12.1, consists of four exons ranging in size from 122 bp to 179 bp. Interestingly, K-*ras* alternatively has four exons (named 4A and 4B) as well, separated by a fragment of nearly 5600 bp [3]. It encodes a 21-kDa protein (named p21^{ras}), localized at the inner surface of the cell membrane, transmitting extracellular signals to a cytoplasmatic cascade [5]. p21^{ras} exists in two physiological states, either in an inactive state (bound to GDP) or, alternatively, in an active state, that requires bound GTP. The conversion of ras-GDP to ras-GTP is catalyzed by guanine nucleotide exchange factors (GEFs, Sos1/2, rasGRF1/2, rasGRP, CNRandGEF), whereas GTPase-activating proteins (GAPs: p120 GAP, NF1-Gap) facilitate the return of active ras to a GDP-bound state [6]. After receiving extracellular stimuli, p21^{ras} interacts with a diverse spectrum of downstream components (Raf seronine/threonine kinase, phosphoinositine 3-phospatase lipid kinase and/or GEFs family of effectors), which initiate a multitude of intracellular signaling pathways [5]. However, activation of *ras* genes by point mutations results in a persistent p21^{ras}, leading to insensitivity for the GAP-stimulating GTP-hydrolysis. In general, aberrant activation of ras proteins may promote uncontrolled proliferation and growth transformation resulting in tumor development [4].

Activation of the K-*ras* gene occurs at specific "hot-spot" regions localized at exon 1 (codons 12 and 13) and exon 2 (codons 59, 61 and 65) in particular [7]. Recently, Wang *et al.* [8] identified K-*ras* gene alterations at codons 15, 27 and 30 in colorectal carcinomas obtained from Taiwanese patients. In human ovarian carcinomas, K-*ras* exon 1 activation appears in up to 85% of cases studied, although the overall incidence of gene alterations, in general, depends on the histological type of ovarian tumors [1, 9]. For example, the fractions of ovarian tumors displayed by K-*ras* gene mutations were significantly higher in mucinous compared to serous and clear-cell carcinomas [10-15]. Interestingly, Mok *et al.* [16] found K-*ras* gene activation in 48% (21 of 44) of borderline ovarian tumors, suggesting that this genetic alteration may be a frequent event in ovarian tumors of borderline malignancy. Teneriello *et al.* [17] believe that K-*ras* gene alterations distinguish low malignant potential (LMP) tumors from invasive ovarian carcinomas. Although K-*ras* gene point mutations have been detected previously, their frequency was low in benign cystadenomas and in LMP ovarian mucinous tumors, supporting the concept of an "adenoma-carcinoma sequence" in the development of mucinous ovarian tumors [18].

This study was undertaken to screen for the existence of K-*ras* gene point mutations in benign and malignant

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ovarian tumors isolated from archival paraffin-embedded slides, including 30 primary human ovarian adenocarcinomas. Regarding the cases of ovarian carcinoma, we additionally assessed p21^{ras} immunostaining for a possible link between the activation of K-*ras* gene and related protein expression.

Material and Methods

Study group

A total of 40 ovarian tumors were randomly chosen from the archive files stored at the Department of Pathology, University School of Medicine, Lublin, Poland. All slides were carefully reviewed by a highly experienced pathologist (E.K.) to confirm the primary diagnosis and to select the appropriate area (above 50% of tumor cells) for DNA isolation. All the patients were primarily diagnosed and operated on at the 2nd Department of Gynecology, University School of Medicine, Lublin, Poland, between 1998 and 2001. Histological classification of tumors was carried out according to the WHO staging system [19]. Included were benign mucinous cystadenomas (n = 2), LMP mucinous tumors (n = 2), ovarian adenocarcinomas (n = 30), adenocarcinoma metastatic to the ovary (n = 1), dysgerminoma (n = 1), folliculoma (n = 2), androblastoma (n = 1), and fibrothecoma (n = 1). Regarding the histologic type of 30 ovarian adenocarcinomas, ten cases were mucinous adenocarcinomas, ten tumors were serous carcinomas, and ten cases were of endometrioid histology.

Clinical staging was established on the basis of the FIGO criteria. All sex cord-stromal cell tumors and germ cell tumors were at clinical Stage I of the disease. Concerning the clinical stage of the ovarian adenocarcinomas, seven (23%) were Stage I, nine (30%) were Stage II, and 14 (47%) were Stage III.

One case of metastatic ovarian adenocarcinoma of mucinous histology, which originated from the mucinous ventricular adenocarcinoma resected one year before, was also included.

DNA isolation-PCR-RFLP

Hematoxylin and eosin slides were carefully microscopically examined, and fields with tumor cells were gently marked. Furthermore, these fields were scraped from four unstained, uncovered 4-μm-thin slides with a razor blade to a sterile eppendorf tube. DNA was extracted using the GenElute™ Mammalian Genomic DNA Kit (SIGMA, USA) according to the manufacturer's instructions.

A fragment of the 157 bp (base pairs) of the exon 1 of the K-*ras* gene was amplified by PCR, as described previously [20, 21], with minor modifications. Briefly, 0.2 ng DNA was amplified in a 50 μl reaction mixture consisting of 200 umol/l each dNTPs, 1.75 mM $MgCl_2$ and 10x *Taq* polymerase buffer (MBI Fermentas, Lithuania). After the initial 5-min step at 95°C, 2.5*U* of *Taq* polymerase DNA (MBI Fermentas, Lithuania) was added ("hot-start" PCR). Thirty-five cycles of amplification consisted of denaturation (30sec. at 94°C), annealing (40sec. at 54°C), and extension (30sec. at 72°C). After the final extension step at 72°C for 10 min, the samples were centifuged and separated by electrophoresis on 2% agarose gel. Following electrophoresis, gel was stained with ethidium bromide and examined by UV light transilluminator (Biometra, Germany) to confirm successful amplification. To avoid intra-laboratory contamination, a control sample without DNA was simultaneously processed in each independent PCR experiment.

Point mutations at exon 1 of the K-*ras* gene were detected by RFLP analysis, incubating the PCR-products with the appropriate enzymes (20 *U* of *MvaI* or 8 *U* of *HphI* for codons 12 or 13, respectively; MBI Fermentas, Lithuania). When a point mutation at either the first or second position of codon 12 of the K-*ras* gene occurred, *MvaI* restriction endonuclease would cleave once (143 bp and 14 bp). However, the wild-type K-*ras* gene fragment was digested enzymatically into three fragments of 114, 29 and 12 nucleotides, respectively.

For detection of point mutations at codon 13 of the K-*ras* gene, a 157 bp fragment is cut by restriction endonuclease *HphI*. The substitution of glycine for aspartic acid created a sequence (GGTGA) which is recognized by *HphI*. When the point mutation at codon 13 occurred, the endonuclease digested the 157 bp fragment into bands of 114 and 43 bp.

Digested PCR fragments were separated by electrophoresis on 8% or 10% polyacrylamide gel, subjected to ethidium bromide staining, and photographed on an ultraviolet light transilluminator. Positive (endometrial cancer harboring K-*ras* point mutations [20]) and negative (human placenta negative for K-*ras* alterations) controls were carried out simultaneously in all experiments. To ensure reproducibility, all positive cases were processed by PCR-RFLP twice.

p21^{ras} immunostaining

Thirty primary human ovarian adenocarcinomas were immunohistochemically stained for the p21^{ras} expression based on the previously described protocol [22]. Briefly, we applied monoclonal mouse anti-human p21^{ras} IgG1 antibody (clone NCC-RAS-001, DAKO, Carpenteria, CA), diluted 1:100, the Envision® Kit (DAKO, Carpenteria, CA), and 3,3''-diaminobenzidine tetrahydrochloride as a chromogen. Simultaneously, positive (endometrial cancer showing an intense positive cytoplasmic signal [22]) and negative (the primary antibody was replaced by control IgG1 antibody from DAKO, Carpenteria, CA) controls were processed. The distribution of specific staining was evaluated by two of the researchers without any knowledge of the clinicopathological variables of cancer. Immunohistochemical reactivity for p21^{ras} was arbitrarily quantified as: (–) less than 10% of tumor cells were positive; (+) 10-50% of tumor cells were positive (expression), and (++) over 50% of tumor cells stained positively (overexpression). Positive staining was considered in that over 10% of tumor cells displayed cytoplasmic staining with anti-p21^{ras} antibody without considering staining intensity (diffuse or focal). Stromal cells (leucocytes, macrophages) were treated as an internal positive control according to Miturski *et al.* [23].

Statistical analysis

Differences in the frequency of K-*ras* activation between groups (regarding patient age, clinical stage and histological features) were investigated by the χ-square test or Fisher's exact test when appropriate; $p < 0.05$ was considered statistically significant.

Results

Molecular alterations of the K-*ras* gene were investigated in the randomly selected group of ovarian tumors, including benign, pre-neoplastic and neoplastic cases, as well as in the uncommon ovarian tumors to test the role of gene activation in the development of these malignancies.

In general, K-*ras* exon 1 point mutations were identified in nine of 40 (22.5%) ovarian tumors selected for this study. K-*ras* codon-12 mutations were detected in none of two benign mucinous cystadenomas, in one out of two (50%) mucinous tumors of LMP, and in five (three muci-

nous, one endometrioid and one serous) out of 30 (17%) primary ovarian adenocarcinomas. Clinical and pathological features of ovarian cancer patients exhibiting K-*ras* gene activation are summarized at Table 1. It is worth pointing out that the 143 bp and 114 bp band patterns were simultaneously identified in all positive cases, suggesting a co-existence of mutant-type with wild-type alleles (Figure 1). Although the frequency of K-*ras* alterations was higher in mucinous than in non-mucinous tumors (30% vs 10%), this difference did not reach significance ($p > 0.05$). No correlation was observed between the clinicopathological features of cancer and the frequency of K-*ras* point mutations in ovarian adenocarcinomas (data not shown). Metastatic ovarian adenocarcinoma of mucinous differentiation, resected from the women who underwent surgery for gastric cancer a year before, showed K-*ras* codon 12 point mutations. $p21^{ras}$ oncoprotein was also expressed in this case (data not shown).

Table 1. — *Clinical and pathological features of ovarian cancer patients harboring the K-ras gene point mutations.*

No.	Age (years)	Histological type	Histological grade	FIGO stage	K-*ras* point mutation codon 12	codon 13	$p21^{ras}$ expression
4	57	Mucinous	G2	IIa	+	–	++
11	64	Endometrioid	G2	IIIa	+	–	++
18	44	Serous	G3	IIb	+	–	+
24	47	Mucinous	G3	IIa	+	–	++
25	40	Mucinous	G2	Ib	+	–	–

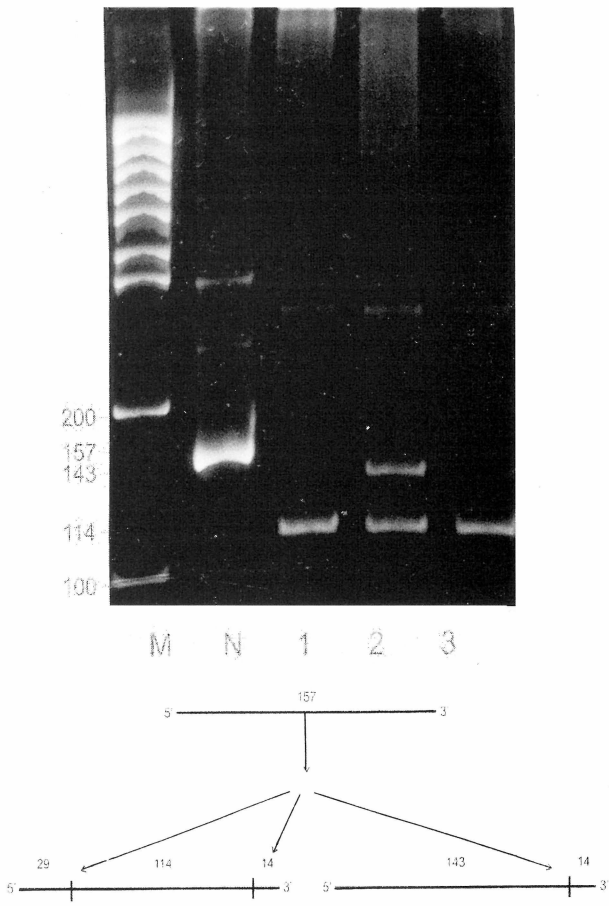

Figure 1. — Detection of K-*ras* codon 12 point mutations by PCR-RFLP. A fragment of 157 bp of exon 1 was amplified, digested and visualized on 10% polyacrylamide gel electrophoresis/ethidium bromide staining. M, Molecular weight marker ("100 bp DNA ladder", MBI Fermentas, Lithuania). N, a 157 bp PCR-product of K-*ras* gene; 1 and 3, ovarian carcinomas negative for the K-*ras* codon 12 point mutations; 2, ovarian adenocarcinoma showing wild-type (114 bp) and mutant-type (143 bp) alleles.

K-*ras* codon 12 point mutations were also reported in one out of four (25%) sex cord-stromal cell tumors (folliculoma). Interestingly, molecular analysis of DNA extracted from the dysgerminoma simultaneously displayed the mutant-type as well as the wild-type alleles, confirming the existence of a point mutation at exon 1 of the K-*ras* gene.

None of the ovarian tumors exhibited K-*ras* codon 13 point mutations.

Cytoplasmic $p21^{ras}$ oncoprotein was expressed (n = 18) or overexpressed (n = 7) in all ovarian adenocarcinomas negative for K-*ras* point mutations (Figure 2A; negative control slide - Figure 2B). Four out of five (80%) K-*ras*-positive ovarian carcinomas expressed $p21^{ras}$ immunohistochemically (Table 1). One tumor (mucinous adenocarci-

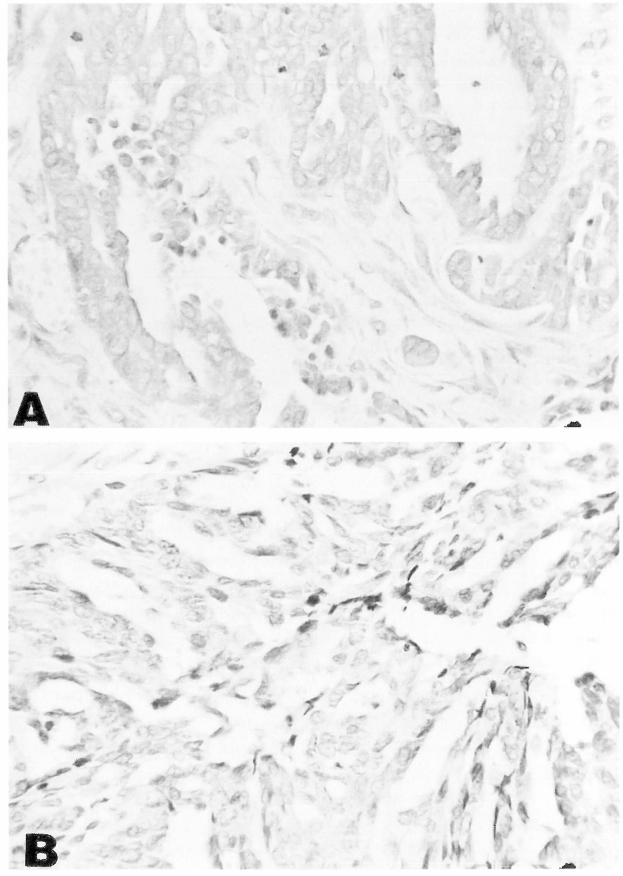

Figure 2. — **A.** Cytoplasmic $p21^{ras}$ oncoprotein was expressed in the epithelial cells of ovarian adenocarcinoma. **B.** Negative control slide (original magnification x 200).

noma presented at Stage Ib of the disease) lacked immunostaining in the cancer nests, although stromal cells were immunoreactive for p21[ras] (internal positive control).

Discussion

Molecular studies of the multitude of human tumors and cell lines identify a variety of oncogenes and TSGs, the alterations of which are undeniably involved in uncontrolled cell division and in the ability to spread or metastasize [3]. K-*ras* is one of the oncogenes, whose derailments are implicated in tumorigenesis in various human organs and cell lines [7]. In our previous study, PCR-RFLP analysis revealed that 14%-15% of endometrial carcinomas contained K-*ras* codon 12 point mutations [20, 24], and we demonstrated a trend towards a worse outcome of endometrial cancer patients harboring K-*ras* activation [20]. Direct sequencing analysis revealed K-*ras* codon 61 point mutations (CA<u>A</u>→CA<u>C</u>, Gln→His) only in two of the 82 (2.4%) human endometrial carcinomas tested [25]. Recently, Stenzel and *co-workers* [21] reported on a coexistence of K-*ras* exon 1 point mutations with "high-risk" HPV infection in 9.7% of human cervical carcinomas, mainly in a subset of moderately differentiated tumors. Studies of K-*ras* point mutations in our laboratory demonstrated gene alterations in a subset (17%) of ovarian adenocarcinomas, with a higher incidence in mucinous than in non-mucinous tumors. Although our patient group was too small to allow revelation of a statistically significant difference between histological subgroups, a relatively higher frequency of K-*ras* alterations has been previously identified in epithelial ovarian carcinomas of mucinous differentiation than in non-mucinus tumors, in particular [10, 18, 26-27]. K-*ras* activation was also detected in mucinous LMP ovarian tumors, which is in line with previously published reports [13, 18, 28]. For example, investigating mucinous ovarian tumors, Cautrecasas *et al.* [13] found K-*ras* codon 12 point mutations in 55.7% of cystadenomas, in 73% of LMP tumors, and in 85% of ovarian carcinomas. Haas *et al.* [28] detected K-*ras* codon 12 point mutations in seven out of 20 ovarian tumors of low-malignant potential, and in two out of six well-differentiated ovarian carcinomas of serous differentiation. However, they reported lack of K-*ras* gene activation in serous cystadenomas or in poorly differentiated ovarian serous carcinomas [28]. Mandai *et al.* [18] noted K-*ras* activation in two LMP portions and in one benign portion of the same case, whereas in another tumor, only LMP and malignant portions, but not benign ovarian epithelium, showed genetic alterations. Moreover, seven ovarian tumors (one benign cystadenoma, three LMP tumors and three carcinomas) displayed K-*ras* gene alterations in all of the portions tested, irrespective of their histological differentiation [18]. The above-mentioned reports as well as our own observations allow us to hypothesize that the LMP tumors and ovarian adenocarcinomas of mucinous differentiation are a continuous spectrum during tumorigenesis. However, *de novo* carcinogenesis from ovarian surface epithelium to mucinous ovarian adenocarcinoma should not be excluded [18]. Recently, Singer *et al.* [29-30] divided ovarian serous carcinomas into two types: low-grade (invasive micropapillary serous carcinoma; MPSC) and high-grade (conventional serous carcinoma; CSC) tumors, depending on various genetic events. However, only MPSC carcinomas were characterized by frequent K-*ras* point mutations, suggesting the influence of a *ras* genetic event on the development of these specific tumors [29]. Finally, comparative analysis of *TP53* and K-*ras* alterations in eight patients, who first presented with advanced-stage serous borderline ovarian tumors and later developed grade 1 serous carcinomas, revealed that borderline and subsequent serous carcinomas are unrelated tumors [31].

A recent study by Gemignani *et al.* [32] assessed the role of K-*ras* and *BRAF* gene mutations in ovarian carcinomas of different histogenesis. Mutations at the K-*ras* gene occurred more frequently in mucinous than in non-mucinous tumors ($p < 10^{-7}$), but none of the mucinous ovarian carcinoma revealed point mutations at the *BRAF* gene. This findings suggest that K-*ras* mutational activation is common in ovarian cancers of mucinous histology, but genes other than *BRAF* are probably implicated in the development in those ovarian tumors negative for K-*ras* activation [32].

Previous reports revealed that p21[ras] expression seems to be associated with the malignant phenotype and the metastatic ability in human solid tumors [33]. In a study conducted by Yaginuma *and co-workers* [34], different ovarian tumors displayed a variable staining intensity for p21[ras], being higher in serous and mucinous adenocarcinomas, undifferentiated adenocarcinomas, and in mature teratomas. The *Western blot* disclosed a statistically significant difference for p21[ras] expression in malignant compared to benign ovarian tumors and in omental metastases compared to ovarian carcinomas, although the staining pattern was not related to either the clinical or the pathological parameters examined [35]. Currently, p21[ras] was expressed in all, except one, human ovarian adenocarcinomas tested. However, lack of protein expression cannot be due to mutations at the "hot-spot" areas alone, but may be caused by gene rearrangements or post-translational protein modification. It is still acceptable that an increased level of p21[ras] is particularly associated with the molecular alterations within the *ras* genes [5]. In contrast, immunohistochemically detectable expression of p21[ras] was not related to K-*ras* gene point mutations in latent prostatic carcinomas [36] or in colorectal carcinomas complicating ulcerative colitis [37]. Moreover, expression of p21[ras] protein without *ras* point mutations seems to occur frequently in human gastric carcinomas (30 out of 45 cases; 66.7%), especially in cases of intestinal subtype [38]. Therefore, overexpression of p21[ras] does not necessarily require the existence of K-*ras* point mutations, as normally overexpressed alleles may also provide a tumorigenic ability [7].

In conclusion, these findings confirm the role of K-ras activation in mucinous ovarian tumors. p21[ras] expression is not necessarily associated with K-*ras* gene alterations in human ovarian adenocarcinomas.

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