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In serous ovarian neoplasms the frequency of Ki-ras mutations correlates with their malignant potential

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Abstract We analysed 44 tissue samples from serous ovarian neoplasms of different malignant potential for *Ki-ras* mutations by denaturing gradient gel electrophoresis (DGGE) and direct sequencing after microdissection. Point mutations at codon 12 were found in 7 of 20 tumours of low malignant potential (LMP) (35%) and in 2 of 6 well-differentiated carcinomas (33%). In contrast, no mutations were detected in the 11 poorly differentiated ovarian carcinoma samples or in the 7 serous cystadenomas. The frequency of Ki-ras mutations in serous ovarian tumours seems to correlate with the malignant potential of the neoplasms. The data favour the hypothesis of a de novo development of poorly differentiated ovarian carcinomas and do not support an evolution from LMP tumours or well-differentiated carcinomas.

Key words *Ki-ras* mutations · Serous ovarian neoplasms · DGGE · LMP tumours · Carcinomas · Cystadenomas

Introduction

The Ras proteins, which are low-molecular-weight GTPases, function as components of the signal transduction pathways that regulate differentiation and proliferation by extracellular stimuli. Normal ras signalling can be disturbed by mutations of the *ras* genes. Point mutations, usually concerning codons 12, 13 or 61, result in persistent activation of the Ras proteins. They have been found in a variety of cancers with different frequencies, depending on the type of tumour [15]. Which of the three *ras* genes (*Ki-*, *Ha-* or *N-ras*) is preferentially mutated is also dependent on the tumour type and in ovarian tumours mutations most often involve codon 12 or 13 of Ki-*ras* [5, 6, 9]. The frequency of these *Ki-ras* mutations depends strongly on the histological type of the ovarian neoplasm: in general the fraction of neoplasms with mutations is higher in mucinous than in serous tumours [5, 6, 9, 12]. In contrast, *Ki-ras* studies comparing tumours of low malignant potential (LMP) and carcinomas of the same histological type show variable results [1, 3]. Thus, it is not clear whether *Ki-ras* mutations are associated with different malignant potential and biological behaviour of ovarian neoplasms. Molecular genetic distinction between the various forms of ovarian cancer may provide valuable insights into the molecular pathways underlying their development and might have an impact on treatment decisions. Therefore, in our study the emphasis was on the comparison between serous ovarian LMP tumours and serous ovarian carcinomas of various grades of differentiation. Additionally, several serous cystadenomas were also analysed for *Ki-ras* mutations. We microdissected the samples and determined the frequency of *Ki-ras* mutations at codons 12 and 13 by denaturing gradient gel electrophoresis (DGGE) and sequencing.

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Materials and methods

The study material comprised a total of 44 samples of ovarian neoplasms. These included 7 serous cystadenomas, 20 serous tumours of LMP, 5 well-differentiated serous carcinomas, 10 poorly differentiated serous cacinomas, and 1 tumour each with well- and poorly differentiated components. The average age of the patients with cystadenomas was 57±20 years, that of those with LMP tumours 51±16 years, and that of those with carcinomas was 65±13 years. Using the criteria of the International Federation of Gynecology and Obstetrics (FIGO) both the LMP tumours and the carcinomas ranged from FIGO I to FIGO III in stage (Table 1). The neoplastic tissue had been fixed in buffered formalin and embedded in paraffin during routine laboratory procedures.

Before the isolation of DNA, the tumour material was microdissected: 5-μm sections were mounted on lysin-coated slides, deparaffinized in xylol and graded ethanol, and briefly stained with methyl green. Tumour areas were identified by comparison with neighbouring sections that had been stained with haematoxylin-eosin. After microdissection under the microscope, between one

Table 1 Number and percentage of identified *Ki-ras* mutations for samples of each of the various types of serous ovarian tumours

Cystadenomas	0/7	(0%)
LMP tumours	7/20	(35%)
Well differentiated carcinomas	2/6	(33%)
Poorly differentiated carcinomas	0/11	(0%)
Total	9/44	(20%)

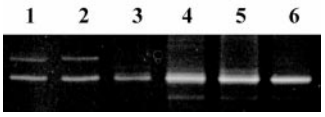

Fig. 1 Vertical denaturing gradient gel electrophoresis of *Ki-ras*-specific PCR product of diverse serous ovarian neoplasms. *Lanes 1, 2* ovarian tumours with *Ki-ras* mutation; *lanes 3–5* ovarian tumours without *Ki-ras* mutation; *lane 6* wild-type control

and three tumour areas from each case were transferred into small tubes and digestion buffer was added (1 mg/ml proteinase K in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Tween-20). After the tissue had been digested for 2 days at 50°C the DNA was extracted with phenol-chloroform-isoamylalcohol followed by one extraction with chloroform-isoamylalcohol.

For DGGE, exon 1 of *Ki-ras* was amplified by PCR in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each deoxynucleotide trisphosphate (Amersham Pharmacia), 0.2 pmol/µl of each primer and 0.025 U/µl AmpliTaq Gold (Perkin Elmer). The primer sequences have been described [7]. PCR conditions were as follows: 9 min at 94°C followed by 40 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. After the final cycle another 8 min at 72°C was added. The PCR products were then run on a 10% polyacrylamide gel with a vertical denaturing gradient from 20% to 80% (100% denaturant corresponds to 7 M urea and 40% formamide). Electrophoresis was performed on a DCode System (BioRad) at 60°C with constant voltage (150 V) for 6 h. Finally, the gel was stained with ethidium bromide and evaluated on a UV screen.

For sequencing, the DNA was amplified under the same PCR conditions as described for DGGE with primers described by Sarkar et al. [11]. The PCR products were purified with a PCR purification kit (Quiagen). After cycle sequencing with a dye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions the sequences were analysed on an ABI PRISM 310 genetic analyser (Applied Biosystems). For each sample up and down sequences were determined.

Results

Analysis by DGGE revealed a band pattern characteristic for point mutations in *Ki-ras* in 9 of the 44 (20%) samples (Fig. 1). This result was confirmed by sequencing of the PCR products after another independent amplification of exon 1 of the *Ki-ras* gene (Table 1). No mutations were found in any of the 7 serous cystadenomas analysed. In 7 of the 20 serous ovarian tumours of LMP (35%) a mutation of codon 12 of the *Ki-ras* gene was detected (Table 2). Four of these muations were GGT → GTT transversions and three were GGT → GAT transitions. The mutations were found in 6 of the FIGO I tumours and in 1 of the FIGO II tumours.

The 6 well-differentiated carcinomas included 2 (33%) that were mutated at codon 12, one having a GGT → GTT transversion and 1 GGT → GAT transition. Both these carcinomas were staged as FIGO III. No mutations were found in the poorly differentiated carcinomas. Case 29, which contained well-differentiated and poorly differentiated areas, was found to have no mutation in the *Ki-ras* gene when each of the tumour components was analysed separately.

No correlation was found between patients' age and frequency of *Ki-ras* mutations.

Table 2 Ki-ras mutations of the individual serous ovarian tumours (*wt* wild type)

Case no.	FIGO	*Ki-ras* mutation
Serous cystadenomas		
1		wt
2		wt
3		wt
4		wt
5		wt
6		wt
7		wt
Serous LMP tumours		
8	I	wt
9	I	wt
10	I	GGT → GTT
11	I	wt
12	I	wt
13	I	wt
14	I	wt
15	I	wt
16	I	GGT → GTT
17	I	wt
18	I	wt
19	I	GGT → GAT
20	I	GGT → GAT
21	I	wt
22	I	GGT → GTT
23	I	wt
24	I	GGT → GAT
25	I	wt
26	II	GGT → GTT
27	III	wt
Well differentiated carcinomas		
28	I	wt
29	II	wt
30	III	GGT → GAT
31	III	wt
32	III	wt
33	III	GGT → GTT
Poorly differentiated carcinomas		
34	I	wt
29	II	wt
35	II	wt
36	II	wt
37	II	wt
38	III	wt
39	III	wt
40	III	wt
41	III	wt
42	III	wt
43	III	wt

Follow-up information was available for 19 of the 20 patients with tumours of LMP. All individuals were alive between 6 months and 7 years after surgery with no evidence of disease. Detailed family histories were available for 19 patients and showed that 2 (cases 17 and 20) had a first- or second-degree relative with cancer (1 breast carcinoma, 1 lung carcinoma). A *Ki-ras* mutation was detected for case 17. Of the 6 carcinoma patients with follow-up data 2 (cases 38 and 41) were alive 3 years after surgery; the other 4 died between 1 and 3 years postoperatively.

Discussion

The *Ki-ras* oncogene is implicated in tumourigenesis in various organs (e.g. [13, 16]). Interestingly, in ovarian neoplasms the frequency of activated *Ki-ras* genes seems to differ considerably, depending on the histological type and the malignant potential of the particular tumour. Therefore, we investigated *Ki-ras* mutations in various forms of serous ovarian tumours, which are the most frequent epithelial neoplasms of the ovary. Our intention was to define a possible correlation between molecular genetic characteristics and phenotype (malignant potential and/or histological type) of the ovarian neoplasms, in order to help elucidate the molecular pathways of carcinogenesis for the diverse entities that ovarian tumours represent. In this context the pivotal question arises of whether carcinomas develop from LMP tumours in a sequential manner or de novo, without LMP tumours as precursor lesions. A deeper understanding of the molecular basis of the phenotypic variability of ovarian tumours is likely to have an impact on the therapeutic approach used in treating these neoplasms. For ovarian neoplasms of the mucinous type similar frequencies of *Ki-ras* mutations have been found in a number of studies (e.g. [2, 5, 10]). In contrast, for ovarian neoplasms of the serous type there are conflicting results in the literature; they range from a clearly higher frequency of *Ki-ras* mutations in LMP tumours than in carcinomas [1, 14] through similar frequency in both forms and even to higher frequencies in carcinomas [6, 8–10]. This may be due in part to the different techniques applied for the mutation analysis (these include RFLP-PCR [3], enrichment PCR [1] or SSCP, and sequencing [8]). We used the very sensitive method of DGGE and direct sequencing of the PCR products combined with microdissection. Some of the series of serous ovarian neoplasms in which we performed screening for *Ki-ras* mutations had been analysed by interphase cytogenetics [4]. From our results the frequency of *Ki-ras* mutations depended strongly on the malignant potential of the neoplasms analysed: Whereas 35% of the LMP tumours showed *Ki-ras* mutations, no mutation at codon 12 or 13 of the *Ki-ras gene* was found in poorly differentiated carcinomas or in serous cystadenomas. These results are in agreement with those recorded in a study by Teneriello et al. [14], who detected *Ki-ras* mutations in 3 of 11 serous LMP tumours but in none of 13 serous carcinomas or 11 serous cystadenomas. In addition, Chenevix-Trench et al. [1] found *Ki-ras* mutations in 3 of 13 serous LMP tumours, in 3 of 30 serous carcinomas and in none of 4 serous cystadenomas. These observations suggest that *Ki-ras* mutations do not have any role in the development or progression of poorly differentiated serous ovarian carcinomas. In contrast, activation of the *Ki-ras* oncogene may well be relevant for the development of a subgroup of serous LMP tumours. Two of the well-differentiated carcinomas were mutated at codon 12 of *Ki-ras*. Thus, the overall impression is that *Ki-ras* mutations are found predominantly in those serous neoplasms that exhibit prominent papillary architecture. For serous ovarian tumours the results do not support the assumption of a progression of serous ovarian LMP tumours to poorly differentiated carcinomas. Rather, a de novo development of highly malignant ovarian carcinomas seems to be probable. Progression of some LMP tumours to well-differentiated serous ovarian carcinomas may be more likely, but this needs to be confirmed in a larger study.

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