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RESEARCH

Molecular Characterization of 103 Ovarian Serous and Mucinous Tumors

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Abstract The pathogenesis of ovarian carcinomas is heterogeneous, with even the same entities showing great variance. In our study we investigated the mutations of the BRAF, KRAS, and p53 genes in serous and mucinous borderline tumors and in low grade and high grade serous and mucinous tumors. The mutations of BRAF and KRAS genes have been shown in 60% of borderline and low grade (well differentiated) serous and mucinous tumors, but very rarely in high grade (moderately and poorly differentiated) carcinomas. However mutations of p53 are very common in high grade tumors and this indicates a "dualistic" model of ovarian tumorigenesis. A total of 80 serous tumors, including serous borderline, low grade and high grade tumors, and 23 mucinous tumors, including borderline and invasive tumors were analysed for BRAF and KRAS mutations using real time PCR method followed by melting point analysis. P53 mutation was investigated by immunohistochemistry. We assumed mutation of the p53 gene when 100% of tumor cells showed strong nuclear positivity. We observed differences in genetic alterations in the development of the low grade tumors and between low and high grade tumors too. In some bilateral or stage II-III cases we observed differences between the mutation status of the left and right ovarian tumors and between the primary tumor and its implants. In one case in a tumor with micropapillary pattern showing high grade nuclear atypia we could detect mutations in both KRAS and p53 genes. The majority of our mucinous ovarian tumor cases showed a KRAS mutation. We have not found mutations of the BRAF and p53 genes in these cases. We have found as have others, that there is a dualistic pathway of ovarian carcinogenesis. In the majority of cases, low grade epithelial tumors develop in a stepwise manner due to genetic alterations of the members of MAP-kinase pathway; however mutation of the p53 gene is the key event in the development of high grade tumors.

Keywords Borderline · Molecular · Mucinous · Ovary · Pathogenesis · Serous

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Introduction

Ovarian cancers are the most lethal gynaecological tumors. The most common types (90% of ovarian cancers) are epithelial tumors. It was observed in the early 1900s that some of these tumors had a better prognosis, and their courses range between the benign and malignant tumors. The FIGO from 1971, and the WHO from 1973 accepted the borderline category for these tumors by definition atypical epithelial proliferation without invasion [1, 2]. The borderline tumors comprise 10–20% of all epithelial ovarian tumors [3].

A lot of tumors show a stepwise manner of tumorigenesis and have a precancerous phase. The prototype of this is the adenoma-carcinoma sequence of colorectal cancers. The exact pathogenesis of ovarian tumors is unknown until today, but according to the data of literature low—and high-grade carcinomas, in the great majority of cases, arise via different genetic pathways.

These data indicate that low-grade serous carcinomas most probably arise via an ‘adenoma–borderline tumour-carcinoma’ pathway. Typically micropapillary serous bor-

derline tumor (MPSBT) progress to low-grade serous carcinoma (LGSC) via alterations of the RAS–RAF signalling pathway secondary to mutations of KRAS and BRAF genes (type I tumours). In the vast majority of cases high-grade serous carcinomas (HGSCs) arise from surface epithelial inclusion glands (currently there is a competing hypothesis for tubal or secunder Müllerian structure origin of high grade serous carcinomas) due to p53 mutations and BRCA1 or BRCA2 dysfunction (type II tumours) [4–10]. Mucinous carcinomas (MCs) often show KRAS mutation.

The most common ovarian epithelial tumors are serous tumors and the second most common are mucinous tumors [11, 12]. More than half of the low grade serous tumors harbour mutations of either BRAF or KRAS gene. The frequency of KRAS mutation is similar in mucinous tumors. Almost all of these tumors show a transition phase from benign lesion to the low grade carcinoma in regard to both their histological and molecular spectrum. [9, 13, 14]. Borderline serous tumors and low grade invasive serous carcinomas have some similarities in genomic alterations, but a lot of differences exist too. Some borderline tumors never progress, whereas some have the potential to become high grade [3].

The Biological Effect of BRAF and KRAS Mutation in Serous and Mucinous Ovarian Tumors

Both BRAF and KRAS genes are the members of the Mitogen-activated protein kinase (MAP-kinase), also known as extra cellular signal-regulated protein kinase (ERK) pathway, which has an important role in the transmission of numerous growth signals into the nucleus [9, 15–17]. This pathway normally relays extra cellular signals from ligand-bound cell surface tyrosine kinase receptors, like epidermal growth factor receptor (EGFR), HER-2, vascular EGFR, platelet-derived growth factor receptor etc. to the nucleus via a cascade of specific phosphorylation events beginning with activation of RAS, and the activation of RAF after it.

The KRAS is a member of the RAS-family genes (KRAS, NRAS and HRAS). The location of the gene is on the long arm of the chromosome 12 (12p12). The RAS proteins have GTPase activity and bind to the inner leaflet of the plasma membrane. Mutations of the KRAS at codon 12 or 13, rarely 61 are mutually exclusive [18]. Point mutations of the KRAS lead to the loss of GTPase activity and constitutive activation of the protein and its downstream signals.

The BRAF gene encodes a RAF family protein, which is recruited by active RAS to stimulate the MAP-kinase pathway. The location of the BRAF gene is on the short arm of the chromosome 7 (7q34). Most of the mutations of B-RAF are clustered in two regions. 90% of the mutations occurred within or adjacent to the activation segment in exon 15, which protects the substrate binding site. 92% of these are a single substitution of adenine (A) for thymidine (T) at nucleotide position 1796, which converts a valine to a glutamic acid (Val to Glu) at position exon 600 (V600E, formerly named V599E) [17, 19–21]. Mutations were identified less commonly in the G loop (glycin rich loop), in exon 11, which mediates the binding of ATP [19].

KRAS and BRAF mutations are exclusive, and do not occur together in the same tumor, in keeping with their functional relationship [4, 10, 14, 20–22] and lead to constitutive activation of the same pathway [6, 14]. Aberrant signalling of MAP-kinase pathway could promote for example growth factor-independent proliferation, insensitivity to growth-inhibitory signals, inactivation of tumor suppressor genes, stimulation of cellular motility causing invasion or metastases, up regulation of angiogenesis, or induction of multidrug resistance gene [17].

Mutations in either KRAS or BRAF have an equivalent and independent effect on tumorigenesis and occur very early in the development of low grade tumors, and play a "gatekeeper" role [21, 23]. It is thought that mutations of the other components of the MAP-kinase pathway are responsible for the development of BRAF and KRAS negative tumors [6].

P53 Mutation and its Role in Ovarian Tumorigenesis

The above mentioned mutations are not found in high grade (moderately and poorly differentiated) [24, 25] carcinomas, whereas the p53 mutation has been shown in more than 60% of cases [6, 9, 10, 18]. The origin of these tumors is controversial. Until now it was believed that these types of tumors do not have known precursor lesions, and it is thought they may develop de novo from the surface epithelium or from an inclusion cyst. In some cases high grade atypia within the epithelium of the ovarian surface or inclusion cyst, termed ovarian intraepithelial atypia (OIN) may be seen [13, 26]. Results of the last ten years show that high grade tumors probably develope from the tubal epithelium or secunder Müllerian structures via in situ carcinoma-carcinoma way. [25, 27–30]. This group includes high grade serous carcinomas, malignant Mixed Müller tumors and undifferentiated carcinomas [5, 10].

According to a few studies p53 mutation occurs in small, stage I high grade serous carcinomas and epithelial cysts adjacent to the tumor or in the tubal epithel. It suggests that mutation of p53 is an early event in the pathogenesis of these tumors [13, 31]. However p53 mutation in type I tumors is a late event, if it occurs at all and is involved in tumor progression rather than initiation [5]

The Clinical Importance of the Molecular Genetic Alterations

The prognosis and the biological behaviour of the two groups are also different. While low grade carcinomas have an indolent course and better prognosis they are resistant to traditional chemotherapy modalities. High grade carcinomas have a poor prognosis, grow rapidly and metastasize early, but respond well to traditional chemotherapy.

Most borderline ovarian tumors present as stage I lesions, and patients with stage I disease have an excellent prognosis with a five-year survival rate of more than 99%. Borderline tumors in stages II–III have slightly lower survival rates, and stage IV tumors have the worst survival rates with a higher percentage of recurrence, in some cases as an invasive low grade carcinoma. The recurrence can appear 20–25 years after the initial diagnosis. Extra ovarian lesions, implants define the behaviour of the low grade malignant potential tumors. The invasiveness of the implants is the most important prognostic factor of stage II–III serous ovarian tumors [32]. The pathogenesis of implants is controversial, and there are two major hypotheses. The first is the monoclonal pathway. According to it, peritoneal implants shed from the surface of the ovarian tumor analogous to endometriosis or endosalpingiosis, or as a direct metastasis [33]. The outgrowth of these exfoliated cells on the peritoneal surface can be facilitated by the similar microenvironment. It is also possible that these non-invasive lesions are reactive mesothelial cell clusters. The second hypothesis is the polyclonal pathway; and is thought to be a result of a field effect. According to it implants are independent primary tumours, independent primary peritoneal carcinomas [16, 33]. Some investigator found the same allelic imbalance and mutational status of KRAS both in SBT and in the implant supporting the implantation theory [34–36]. Others have shown a different X chromosome inactivation patterns in the SBT and in its implants, which would support the independent origin of the peritoneal lesions [37, 38].

In contrast to the borderline tumors the five-year survival rate for stage I invasive carcinomas is 65–80%, and 29–49% for stage III invasive carcinoma. The borderline tumors have a better prognosis in every stage compared to low grade tumors. The low grade serous carcinomas show an indolent course, but after 20–25 years many patients die due to widespread carcinomatosis. These tumors are resistant to chemotherapy.

Inhibition of the members of MAP-kinase pathway would be a rational therapy against the advanced stage low grade carcinomas showing KRAS or BRAF mutations. These types of anticancer therapy may prove to be more effective, specific and less toxic than conventional cytotoxic agents. There are many drugs in phase II or III clinical trials.

The aim of this study was to investigate the BRAF and KRAS mutation, and p53 expression in borderline, low grade invasive, and high grade invasive, serous and mucinous ovarian tumors; to determine these mutations in extra ovarian manifestations and to compare the molecular background of the primary and secondary lesions.

Materials and Methods

Patients and Paraffin Embedded Samples

103 pathology samples of 87 patients with ovarian tumors (80 serous and 23 mucinous tumours) diagnosed in the National Institute of Oncology between 2000 and 2009 were analysed in this study (between 2000–2009 altogether 43 serous borderline tumors were diagnosed in our institute and we have investigated also 17 low grade and 20 high grade serous carcinomas from the occurring cases. The most of the high grade tumors we could investigate only a part of the tumors because of the inoperability of the disease. 12 mucinous borderline tumors were occurred in our institute and we have chosen 10 mucinous carcinomas too; so these cases don't represent the proportion of their occurrence in the population.)

While the age distribution of the patients with serous borderline tumor (SBT) ranged from 18 to 73 with a median age of 44,3 years, and with low grade serous carcinoma (LGSC) varied from 26 to 77, the median age was 48,76 years. Patients with high grade serous carcinoma (HGSC) were older, and the age distribution ranged from 41 to 78 years with a median age of 61,8 years.

The age of patients with mucinous borderline tumors (MBT) was between 40 and 83 years, and the median age was 55,4 years and with mucinous adenocarcinoma (MC) ranged from 47 to 75 with a median age 60,3 years.

Representative formalin fixed, paraffin embedded blocks were chosen following review of the cases. Tumor typing and staging were performed according to the criteria of the International Federation of Gynaecologists and Obstetricians (FIGO) and the International Union against Cancer (IUCC). In cases of mucinous tumors we ruled out of the metastatic origin by both clinically and immunohistochemically (all cases were CK7 positive and CK20 and CDX2 negative).

All the tumors were reviewed by haematoxylin-eosin and screened for the presence of the most common V600E BRAF mutation in the exon 15 and for KRAS mutations in the codon 12 and 13 by melting point analysis after real time PCR reaction. Results were confirmed with bidirectional sequencing. One sample per patient were analysed,

except for the bilateral cases or stage II–IV tumours, where we analysed both the primary ovarian tumors and the extra ovarian lesions. 98 cases respectively were analysed by p53 immunohistochemistry.

Immunohistochemistry

4 μm sections from each case were used for immunohistochemical detection of p53. The procedures were performed according to the manufacturers' protocol. The sections were deparaffinized and rehydrated in graded alcohol. After heat-induced epitope retrieval (HIER) by steaming in citrate buffer (DakoCytomation Target Retrieval Solution, code No. S 3308, pH 6.0) for 35 minutes) sections were stained with p53 antibody (DAKO clon: DO-7, 1:300, incubation time was 30′ at room temperature). Avidin-biotin complex (ABC) and peroxidase methods were used. The visualization was performed by DAB (3,3′-diaminbenzidine chromogen) using DAKO LSAB/HRP kit and DAKO EnVision/HRP kit. Only cases showing 100% tumor cell positivity were regarded as positive for p53 gene mutation [5].

Mutational Analysis of BRAF and KRAS Genes

Extraction of DNA

Samples were subjected to cell lysis with proteinase K treatment (Magna Pure DNA Tissue lysis, proteinase K, Roche, Germany) followed by DNA extraction using magnetic bead technology (Magna Pure CNA, Roche Diagnostics, Germany) according to the manufacturer's instruction. The purified samples were stored at −20°C in a concentration of 30 ng/μl.

Detection of Kras Mutation with Real-Time PCR Followed by Melting Curve Analysis

A set of primers were chosen to amplify a specific 170-bp genomic fragment of K-ras exon 2 containing codon 12, 13. Hybridisation probes were designed complementary to wild type K-ras allele. The competing wild-type LNA oligomer covered codon 10–14. The antisense strand was chosen for the LNA oligomers and the detection probes because of its lower purine content and, therefore more precise hybridisation results (Li Y). The primers, probes and the LNA oligomers were from TibMolbiol (LightCycler Probe Design Software 2.0, manufactured by TibMolbiol, Germany). The sequences of the primers, probes and LNA oligomers are given in Table 1. The PCR was conducted in a LightCycler 2.0 thermocycler (Roche Diagnostics). The PCR mixture contained 1 μM forward primer, 0.25 μM reverse primer, 0.2 μM each of the probes, 5.5 mM $MgCl_2$, 5× ready-to-use master mix (Lightcycler Multiplex DNA master HybProbe kit from Roche) and 1 μl of DNA template in a total volume of 10 μl. After the initial denaturation step (10 min for 95°C) the conditions of the PCR were 95°C for 5 sec, followed by 60°C for 15 sec, 72°C for 15 seconds with 5°C/s ramp rate in each cycle for 45 cycles.

Mutant and wild type positive control in each run and water as negative control (as control for contamination) were also processed in parallel with each sample.

After the thermal cycling we performed melting curve analysis to detect sequence variations in the amplicons (95°C for 10 sec, 40°C for 30 sec to 95°C for 0 sec with continuous measure on F2 channel (for LC-Red 640 nm) and 0.1°C/s ramp rate).

PCRs for the LNA clamp were run with the same conditions except that we added 0.05 μM LNA oligo, 0.5 μM forward-reverse primers to the basic mixture and set 10 sec at 70°C T_m step before annealing to maximize LNA binding to its complementary DNA strand.

The K-ras gene mutation was identified by comparing the Tm of each patient's results with that of the DNA positive and negative controls.

After detecting sequence variations and purification of PCR products (NucleoSpin extractII, Macherey-Nagel, Germany) results were confirmed with bidirectional sequencing analysis on the ABI Prism Genetic analyzer instrument. (Applied Biosystems).

Table 1 DNA sequences of primers, probes and LNA oligomers for detecting K-ras mutations

K-ras forward:	5′ tcatattcgtccacaaaatgattctg 3′
K-ras reverse:	5′ gtattaaccttatgtgtgacatgttcta 3′
sensor	5′ ac**gccacc**agctccaac-fluorescein 3′
anchor	5′ LC Red 640-ccacaagtttatattcagtcattttcagcaggcct-phosphate 3′
LNA	5′ **CCTACGCCACCAGCTCC**-NH2 3′

Bold lower case: K-ras codon12 and 13

The 3′ end of the anchor probe was phosphorylated to prevent probe elongation by Taq polymerase during PCR. The melting temperature of the probes is 60°C

Detection of Braf Mutation with Real-Time PCR Followed by Melting Curve Analysis

Detection of Braf mutation was done by similar methods as Kras mutation detection.

A set of primers were chosen to amplify a specific 227-bp genomic fragment of Braf exon 15 containing codon 600 [39]. Hybridisation probes were designed complementary to wild type Braf allele. The primers and probes were from TibMolbiol (LightCycler Probe Design Software 2.0, manufactured by TibMolbiol, Germany). The sequences of the primers and probes are given in Table 2.

The PCR was conducted in a LightCycler 2.0 thermocycler (Roche Diagnostics). The PCR mixture contained 1 μM forward primer, 0.25 μM reverse primer, 0.2 μM each of the probes, 4 mM $MgCl_2$, 1x ready-to-use master mix (Lightcycler Multiplex DNA master HybProbe kit from Roche) and 2 μl of DNA template in a total volume of 20 μl. After the initial denaturation step (10 min for 95°C) the conditions of the PCR were 95°C for 5 sec, followed by 50°C for 5 sec 55°C for 15 sec, 72°C for 15 sec with 5°C/sec ramp rate in each cycle for 54 cycles.

Mutant and wild type positive control in each run and water as negative control (as control for contamination) were also processed in parallel with each sample.

After the thermal cycling we performed melting curve analysis to detect sequence variations of the amplicons (95°C for 10 sec, 45°C for 60 sec to 95°C for 0 sec with continuous measure on F2 channel (for LC-Red 640 nm) and 0.1°C/s ramp rate).

The Braf gene mutation was identified by comparing the Tm of each patient's results with that of the DNA positive and negative controls.

After detecting sequence variations and purification of PCR products (NucleoSpin extractII, Macherey-Nagel, Germany) results were confirmed with bidirectional sequencing analysis on the ABI Prism Genetic analyzer instrument. (Applied Biosystems).

Table 2 DNA sequences of primers and probes for detecting Braf mutation

Braf forward:	5′-tcataatgcttgctctgatagga-3′
Braf reverse:	5′-ggccaaaaatttaatcagtgga-3′
sensor	5-agctaca**gtg**aaatctcgatggag-fluoreszcein-3′
anchor	5′-LC Red 640-ggtcccatcagtttgaacagttgtctgga-phosphate-3′

Bold lower case: Braf codon 600

The 3′ end of the anchor probe was phosphorylated to prevent probe elongation by Taq polymerase during PCR. The melting temperature of the probes is 60°C

Results

Table 3 shows the histological distribution and the results of molecular analysis of the reviewed cases. 27 of serous neoplasm were serous borderline tumors (three with microinvasion; seven bilateral; in bilateral cases the contra lateral ovary showed SBT in 4 case, MPSBT in 1 case and LGSC in 2 case). Among 16 borderline tumors with micropapillary features, two showed microinvasion and six were bilateral and in one case the other ovary showed typical serous borderline tumor, and in one case we saw low grade serous carcinoma in the contra lateral ovary. 17 cases were diagnosed as invasive low grade serous carcinoma (seven bilateral; two cases showed typical serous borderline tumor and one case micropapillary serous borderline tumor in the contra lateral ovary, the remaining cases were bilateral serous carcinomas). 20 cases were high grade serous carcinomas. Invasive implants were not seen in cases of borderline tumors, but in 8 cases we detected non-invasive implants. Invasive carcinomas had non-invasive implants in 6 cases and there were invasive implants or metastasis in 8 cases.

12 mucinous tumors were borderline mucinous neoplasm, among these three showed microinvasion, and among them there was a bilateral lesion with a low grade mucinous carcinoma of the other ovary. There were 11 low grade mucinous carcinomas, among them the bilateral case with borderline mucinous tumor.

Transition between benign, borderline and low grade serous carcinomatous areas were seen in the majority of cases of borderline tumours and of low grade serous carcinomas. Benign areas were detected in 23 of the 27 typical borderline tumours. There were also benign and typical borderline areas in 13 of the 16 cases of micropapillary serous tumors. The area of epithelial proliferation was in inverse ratio to the extent of benign areas. The proportion of the adenomatous areas in borderline tumors with micropapillary features were less than 10%.

In the majority of the low grade serous carcinomas borderline areas were seen too, but foci of benign adenoma infrequently. Three cases showed small areas of typical benign cystadenoma besides carcinoma, and in 8 cases micropapillary borderline tumors were detected too. We were unable see any precursor areas in one case.

Precursor lesions were not detected in high grade serous carcinomas at all. We could see neither low grade carcinomatous component, nor borderline or benign areas. Normal residual ovarian tissue was not detected either.

12 borderline mucinous neoplasm contained areas of residual benign cystadenoma and there were borderline areas in 8 of 11 invasive mucinous neoplasm.

Table 3 Histological distribution and results of molecular analysis of the investigated cases

Histological subtype	Unilateral	Bilateral	Microinvasive area	Implant		Mutational status Positive results/No of examined case		
				Invasive	Non-invasive	P53	BRAF	KRAS
SBT 27 cases	16	7 (1 SBT-MPSBT♣ 2 SBT-LGSC► 4 SBT-SBT)	2 unilateral 1 bilateral	0	5	0/43 (0%)	10/43 (23,25%)	17/43 (39,53%)
MPSBT 16 cases	6	6 (1 MPSBT-SBT♣ 1MPSBT-LGSC• 4 MPSBT-MPSBT)	1 unilateral 1 bilateral	0	3			
LGSC 17 cases	6	7 (2 LGSC - SBT ► 1 LGSC- MPSBT• 4 LGSC-LGSC)	–	8	6	0/17 (0%)	0/17 (0%)	4/17 (23,53%)
HGSC 20 cases			–			11/20 (55%)	0/20 (0%)	2/20 (10%)
MBT 12 cases	11	1 (MBT- MC)◊	3			0/12 (0%)	0/8 (0%)	6/12 (50%)
MC 11 cases	10	1 (MC- MBT) ◊				0/6 (0%)	0/6 (0%)	7/11 (63,63%)

♣, ►, •, ◊same patients

SBT-serous borderline tumor, MPSBT-micropapillary serous borderline tumor, LGSC-low grade serous carcinoma, HGSC-high grade serous carcinoma, MBT-mucinous borderline tumor, MC-mucinous carcinoma

Results of Mutation Analysis

We detected BRAF mutation at codon V600E (all mutations were detected in this location) in 10 cases among all 43 borderline serous tumors (Figure 1), and we did not observe it at all in the 17 cases of low grade serous tumors (Figure 2) and high grade tumors. The KRAS mutations in the codon 12 were demonstrated in 17 serous borderline tumors, in 4 low grade carcinomas and in 2 high grade carcinomas respectively. There were no cases where the two gene mutations occurred together. All together the two gene mutations occurred in 63% of the serous borderline tumors, in 24% of the serous low grade tumors, but only 10% of the high grade serous tumors. Among the 20 bilateral cases five showed different genetic alterations of the two ovarian tumors. In all 5 cases one of the ovarian tumors showed KRAS mutation and the contra lateral tumor showed wild type KRAS gene. The remaining bilateral cases showed similar genetic status. We could demonstrate BRAF mutation in both of ovaries in 2 cases and KRAS mutation in 5 cases respectively. The remaining bilateral tumors did not show KRAS or BRAF mutation.

In 12 cases we investigated in parallel the primary ovarian tumor and the peritoneal implants (among them 7 cases were bilateral). We detected the same mutation in the tumors and their implants in 7 cases. In two bilateral cases the two ovarian tumors showed different genetic alterations, one of them was KRAS mutant, and the other was wild type. In these cases the implants were wild type. In one unilateral case the primary ovarian tumor was KRAS mutant but the implant was wild type. In another unilateral case the implant showed KRAS mutation while the ovarian tumor was wild type.

Borderline or low grade serous tumors did not show p53 mutation. In contrast to the borderline serous and low grade invasive carcinomas the high grade serous tumors showed p53 mutation in 55% of cases (11 cases from 20 cases). One case showed both p53 and KRAS mutation. This tumor had a micropapillary component, but the nuclear polymorphism and the mitotic rate were high (Figure 3).

Among the 23 analysed ovarian mucinous tumors we did not find BRAF or p53 mutations. There were KRAS mutation in 6/12 borderline cases, and in 7/11 mucinous carcinomas. Bilateral mucinous cases showed no differences in genetic alterations of the two ovarian tumors (Table 3).

Discussion

Our findings, like previous studies support the hypothesis of the dualistic model of ovarian carcinogenesis. According to it Type I ovarian tumours (including the low grade serous, mucinous, endometrioid and clear cell carcinomas) show a step-wise tumorigenesis, and in cases of Type II tumours (high grade serous carcinoma, mixed Müller tumor and undifferentiated carcinoma) we didn't see any precursor lesion, but in the most cases we could see only a part of the tumors. In Type I tumors the most frequent genetic abnormality is the mutations of the members of MAP-

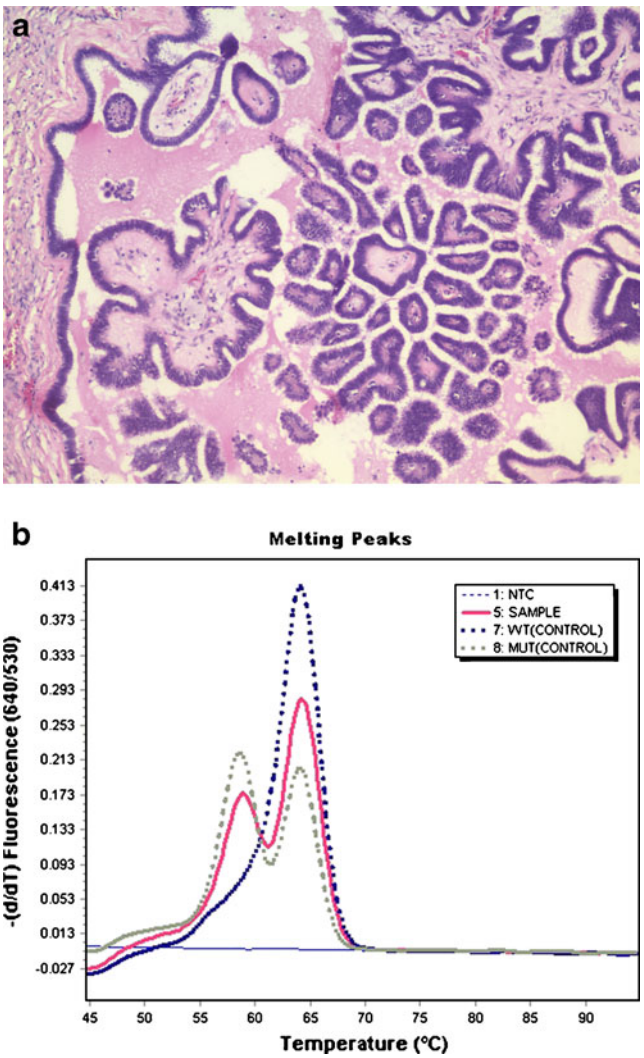

Fig. 1 (**a**) Typical serous borderline tumor (HE 100×). (**b**). The tumor showed mutation of Braf gene. Melting point of the tumor was similar to the mutant control's. (NTC-non template control, WT-wild type, MUT-mutant)

kinase pathway i.e. KRAS and BRAF genes, Type II tumors show mainly mutation of the p53 gene [5, 10, 13]. The morphological spectrum of transition from benign lesions to low grade carcinomas, which can be seen in borderline tumors, low grade serous carcinomas, and mucinous tumors but are not seen in high grade tumors also favours this step-wise tumorigenesis.

As in previous studies we detected BRAF and KRAS mutation in a significant proportion of ovarian borderline and low grade invasive carcinomas. In serous borderline tumors BRAF and KRAS mutations never occur together [4, 10, 19]. We found very similar KRAS mutations in borderline and low grade serous carcinomas suggesting a similar molecular pathway. However we did not seen BRAF mutation in low grade serous carcinomas at all. We could not define clearly the predisposing genetic factors for

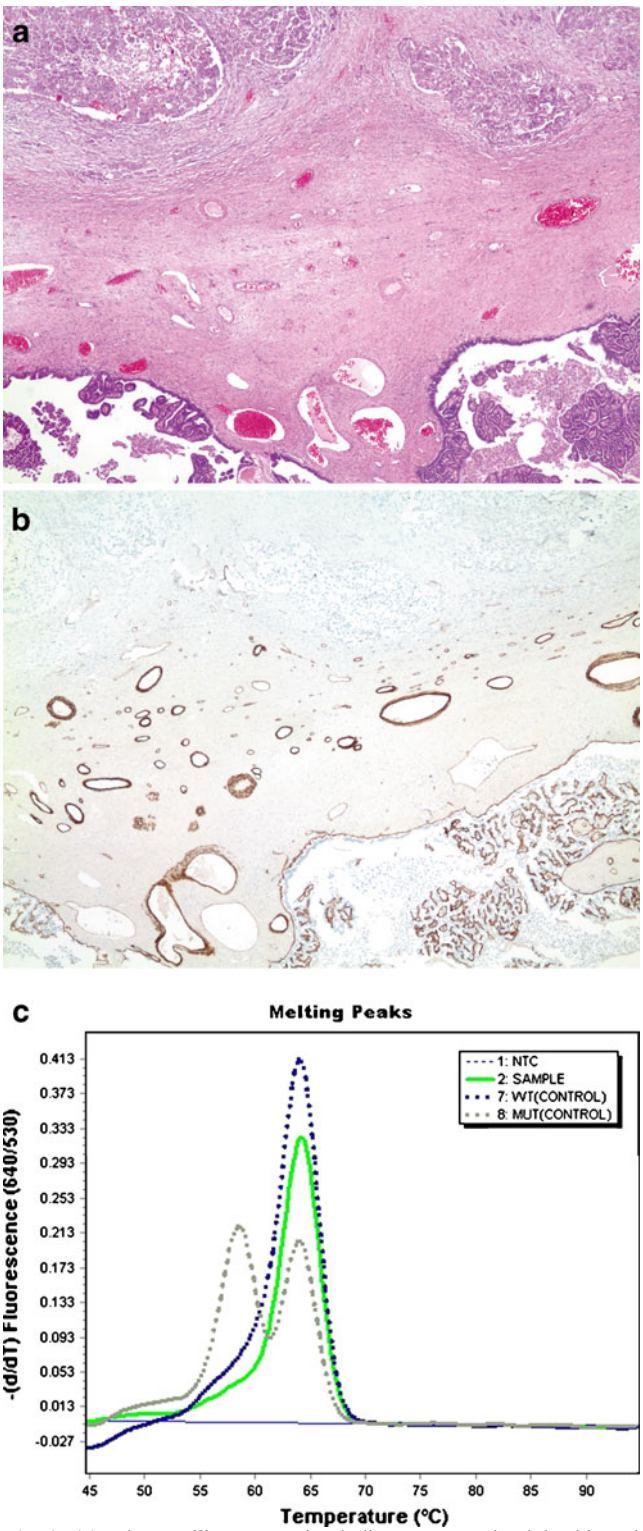

Fig. 2 (**a**) Micropapillary serous borderline tumor on the right side and low grade serous carcinoma on the left side in the same ovary (HE 40×). (**b**). Laminin immunohistochemistry in the same tumor. The basalmembrane is continuous in micropapillary serous borderline tumor (right side). Staining is disappearing in low grade serous carcinoma (left side) (laminin 40×). (**c**). Braf were wild type in both low grade serous carcinoma and the borderline component of the ovarian tumor. Melting point of the tumor sample was the same like wide type control's. (NTC-non template control, WT-wild type, MUT-mutant)

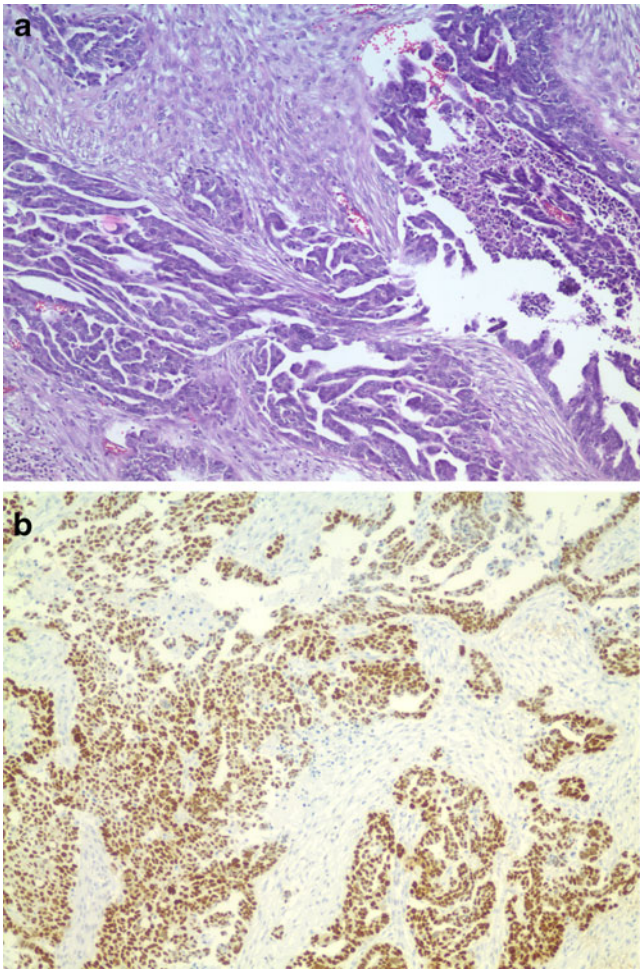

Fig. 3 (**a**) High grade serous carcinoma with micropapillary feature and high grade nuclear polymorphism (HE 40×). (**b**). p53 immunohistochemistry showed nearly 100% positivity of the tumor cells. (K-ras gene was also mutant in this tumor). (40×)

progression of a borderline tumor to a low grade carcinoma, but our results suggest that tumors with borderline morphology and BRAF mutation are more stable and do not progress to low grade serous carcinoma. It seems feasible that the two mutations have different roles in the tumorigenesis. While the BRAF mutation causes only borderline lesions, KRAS mutation causes not only epithelial proliferation, but some disposition to progress to carcinoma. Nonetheless BRAF and KRAS mutations employ biologically similar mechanisms to induce epithelial proliferation, and have different roles in carcinogenesis. It must be mentioned that in the literature the occurrence of KRAS and BRAF mutations in low grade serous carcinomas are nearly equivalent [10, 13, 20, 26, 40]. The reason however could be the low number of cases. Corresponding to previous reports there were no morphological or clinical differences between the BRAF or KRAS positive and negative cases [19]. We detected only KRAS mutation in mucinous tumours which is also in accordance with previous papers [9, 10, 13, 16, 26].

These molecular changes are confined to the first group of ovarian surface neoplasms and occur very rarely in the second group. In the second group, of high grade carcinomas p53 mutations were detected most often. It is not a feature of the low grade tumors.

Like previous studies our results showed that low grade serous carcinomas are unrelated to high grade tumors [19]. However the possibility still exists, that BRAF or KRAS wild type or mutant low grade serous tumors may progress to high grade tumors [10]. It may be that another molecular event is needed to cause progression. We had a case where we could detect p53 and KRAS mutations together in the same tumor which showed micropapillary features, prominent nuclear polymorphism and a high mitotic rate. Most probably it represents that rare example when a low grade carcinoma progresses to a high grade tumor (Fig. 3).

We can conclude that in the majority of the cases of ovarian serous carcinomas, the two different types (low grade with papillary structures, nuclear grade I–II, low mitotic activity;) and high grade (which shows mainly solid, papillary and adenoid structures and high nuclear grade and mitotic activity) [10, 41] are two different tumors.

Our results underline the monoclonal hypothesis of ovarian tumors and their implants as we could detect the same mutations in the bilateral cases and in the extra ovarian lesions in the majority of the cases. But in a few cases there were differences between the molecular status of the bilateral ovarian tumors, or between the ovarian tumor and its implants, so we can not exclude the polyclonal hypothesis with a “field effect”.

Our results suggest different pathways of tumorigenesis of low grade serous tumors. On the basis of our results we can say that KRAS mutation plays an important role in the progression of borderline tumors to low grade carcinomas. Benign serous tumors can progress to borderline tumors due to a BRAF mutation but they do not tend to progress to low grade carcinomas. When borderline tumors do not show a BRAF mutation they progress to low grade carcinomas due to KRAS mutation or some other genetic event.

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References

1. FIGO. Intenational Federation of Gynecology and Obstetrics (1971) Classification and staging of malignant tumours in the female pelvis. Acta Obstet Gynecol Scand 50:1–7
2. Serov SF, Scully RE, Sobin LH (1973) International histologic Classification of Tumours. No. 9. Histological Typing of Ovarian Tumours. World Health Organization, Geneva
3. Österbeerg L, Akeson M, Levan K et al (2006) Genetic alterations of serous borderline tumors of the ovary compared to stage I serous ovarian carcinomas. Cancer Genet Cytogenet 167:103–108
4. Singer G, Oldt R, Cohen Y et al (2003) Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J Natl Cancer Inst 95:484–486
5. Singer G, Stöhr R, Cope L et al (2005) Patterns of p53 mutations separate ovarian serous borderline tumors and low —and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis (A mutational analysis with immunohistochemical correlation). Am J Surg Pathol 29(2):218–224
6. Hsu C-Y, Bristow R, Cha M et al (2004) Characterization of active mitogen-activated protein kinase in ovarian serous carcinomas. Clin Cancer Res 10:6432–6436
7. Staebler A, Diebold J (2007) Molekularpathologie der epithelialen Ovarialneoplasien (Von der Phänotyp-Genotyp-Korrelation zu neuen Ansatzpunkten in Diagnostik und Therapie). Pathologe 28:180–186
8. Ho C-L, Kurman RJ, Dehari R et al (2004) Mutations of BRAF and KRAS precede the development of ovarian serous borderline tumors. Cancer Res 64:6915–6918
9. Shih I-M, Kurman RJ (2004) Ovarian tumorigenesis (Review). Am J Path 164(5):1511–1518
10. Kurman RJ, Shih I-M (2008) Pathogenesis of ovarian cancer. Lessons from morphology and molecular biology and their implications. Int J Gynecol Pathol 27(2):151–160
11. Seidman JD, Horkayne-Szakaly I, Haiba M et al (2004) The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. Int J Gynecol Pathol 23:41–44
12. Hart WR (2005) Borderline epithelial tumors of the ovary. Mod Path 18:33–50
13. Bell DA (2005) Origins and molecular pathology of ovarian cancer. Mod Pathol 18:S19–S32
14. Christie M, Oehler MK (2006) Molecular pathology of epithelial ovarian cancer. J Br Menopause Soc 12(2):57–63
15. Singer G, Kurman RJ, Chang H-W et al (2002) Diverse tumorigenic pathways in ovarian serous carcinoma. Am J Pathol 160:1223–1228
16. Shih I-M, Kurman RJ (2005) Molecular pathogenesis of ovarian borderline tumors: new insight and old challenges. Clin Cancer Res 11:7273–7279
17. Sridhar SS, Hedley D, Siu LL (2005) Raf kinase as a target for anticancer therapeutics. Mol Cancer Ther 4(4):677–685
18. Pohl G, Ho C-L, Kurman RJ (2005) Inactivation of the Mitogen-Activared Protein Kinase pathway as a potential target-based therapy in ovarian serous tumors with KRAS or BRAF mutations. Cancer Res 65(5):1994–2000
19. Sieben NLG, Macropoulos P, Roemen GMJM et al (2004) In ovarian neoplasms BRAF, but not KRAS, mutations are restricted to low-grade serous tumours. J Pathol 202:336–340
20. Mayr D, Hirschmann A, Löhrs U et al (2006) KRAS and BRAF mutations in ovarian tumors: A comprehensive study of invasive carcinomas, borderline tumors and extraovarian implants. Gynecol Oncol 103:883–887
21. Ueda M, Toji E, Noda S (2007) Germ line and somatic mutations of BRAF V599E in ovarian carcinoma. Int J Gynecol Cancer
22. Wang L, Arnold K (2003) Common mutations prevalent in low-grade ovarian cancers. J Nat Cancer Inst 95(6):417–418
23. Garrett AP, Lee KP, Colitti CR et al (2001) K-RAS mutation may be an early event in mucinous ovarian tumorigenesis. Int J Gynecol Pathol 20:244–251
24. Vang R, Shih I-M, Salani R et al (2008) Subdividing ovarian and peritoneal serous carcinoma into moderately differentiated and poorly differentiated does not have biologic validity based on molecular genetic and in vitro drug resistance data. Am J Surg Pathol 32(11):1667–1674
25. Köbel M, Hunstman D, Gilks CB (2008) Critical molecular abnormalities in high grade serous carcinoma of the ovary. Expert Rev Mol Med 10:e22
26. Russell SEH, McCluggage WG (2004) A multistep model for ovarian tumorigenesis: the value of mutation analysis in the KRAS and BRAF genes. L Pathol 203:617–619
27. Lee Y, Miron A, Drapkin R et al (2007) A candidate precursor to serous sarcinoma that originates in the distal fallopian tube. J Pathol 211(1):26–35
28. Goodman MT, Shvetsov YB (2009) Incidence of ovarian, peritoneal, and fallopian tube carcinomas in the United States, 1995–2004. Cancer Epidemiol Biomarkers Prev 18(1):132–139
29. Tong G-X, Chiriboga L, Hamele-Bena D et al (2007) Expression of PAX2 in papillary serous carcinoma of the ovary: immunohistochemical evidence of fallopian tube or secondary Müllerian system origin? Mod Pathol 20:856–863
30. Roh MH, Kindelberger D, Crum CP (2009) Serous tubal intraepithelial carcinoma and dominant ovarian mass: clues to serous tumor origin? Am J Surg Pathol 33(3):376–383
31. Leiato MM, Soslow RA, Baergen RN et al (2004) Mutation and expression of the TP53 gene in early stage epithelial ovarian carcinoma. Gynecol Oncol 93:301–306
32. Morice P, Uzan C, Kane A et al (2009) Prognostic factors of patients with advanced stage serous borderline tumors of the ovary. J Clin Oncol 27:15s (suppl;abstr 5573)
33. Sieben NL, Roemen GMJM, Oosting J (2006) Clonal analysis favours a monoclonal origin for serous borderline tumours with peritoneal implants. J Pathol 210:405–411
34. Krishnamurti U, Sasatomi E, Swalsky PA (2005) Microdissection-based mutational genotyping of serous borderline tumors of the ovary. Int J Gynecol Pathol 24:56–61
35. Diebold J, Seemuller F, Lohrs U (2003) K-RAS mutations in ovarian and extraovarian lesions of serous tumors of borderline malignancy. Lab Invest 83:251–258
36. Zanotti KM, Hart WR, Kennedy AW (1999) Allelic imbalance on chromosome 17p13 in borderline (low malignant potential) epithelial ovarian tumors. Int J Gynecol Pathol 18:247–253
37. Gu J, Roth LM, Younger C et al (2001) Molecular evidence for the independent origin of extraovarian papillary serous tumors of low malignant potential. J Natl Cancer Inst 93:1147–1152
38. Lu KH, Bell DA, Welch WR et al (1998) Evidence for the multifocal origin of bilateral and advanced human serous borderline ovarian tumors. Cancer Res 58:2328–2330
39. Davies H, Bignell GR, Cox C et al (2002) Mutations of BRAF gene in human cancer. Nature 417:949–954
40. Cheng EJ, Kurman RJ, Wang M et al (2004) Molecular genetic analysis of ovarian serous cystadenomas. Lab Invest 84:778–784
41. Burks RT, Sherman ME, Kurman RJ (1996) Micropapillary serous carcinoma of the ovary: a distinctive low-grade carcinoma related to serous borderline tumors. Am J Surg Pathol 20:1319–1330