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Mutation analysis and genomic imbalances of cells found in effusion fluids from patients with ovarian cancer

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Abstract. Ovarian carcinomas and carcinosarcomas often cause malignant effusions, an accumulation within serous cavities of fluid containing cancer cells. Few studies have focused on the molecular alterations and genetic mechanisms behind effusion formation. The present study investigated the mutation status of *TP53*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS* and *BRAF* in effusion fluids from 103 patients with ovarian cancer. In addition, array Comparative Genomic Hybridization (aCGH) analysis was performed on 20 effusions from patients with high-grade serous carcinoma (10 cases positive for *TP53* mutation and 10 with *TP53* wild-type). *TP53* mutations, two of which were novel: c.826_830delCCTGT and c.475_476GC>TT, were identified in 44% of the cases. Mutations in *KRAS*, *HRAS,* and *PIK3CA* were identified in two, two and four cases, respectively. None of the effusions analysed showed *NRAS* or *BRAF* mutations. The aCGH analysis revealed highly imbalanced genomes similar to those described in primary ovarian carcinomas. No specific profile was indicated to distinguish tumors with *TP53* mutations from those without. The molecular profiling of cells found in effusion fluids from patients with ovarian cancer thus showed considerable molecular heterogeneity. *TP53* seems to be the most frequently mutated gene in these cells and may serve a leading role in the metastatic process.

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Introduction

Cancers of the ovaries, most of which are carcinomas (OC), are the eighth most common malignancy in women and the most lethal one. In the year 2018, 295,414 new cases were diagnosed and 184,799 deaths occurred from ovarian cancer worldwide (1). OC can be subdivided into various histological subtypes, each showing distinct genomic and epigenomic characteristics (2). High-grade serous carcinoma (HGSC) is the most frequent and aggressive histotype, comprising 70% of newly diagnosed cases. Less frequent are endometrioid carcinoma (EC, 15%), clear cell carcinoma (CCC, 12%), low-grade serous carcinoma (LGSC, <10%), and mucinous carcinoma (MC, 3%) (3). Carcinosarcomas (CS) of the female genital tract are biphasic tumors containing some areas showing carcinomatous growth, mostly HGSC, and others displaying sarcomatous differentiation. CS are rare but aggressive tumors that often prove fatal within 1-2 years of diagnosis (4).

The majority of malignant ovarian effusions stem from carcinomas or CS (5,6). They are an almost universal clinical finding in advanced-stage OC, i.e., stage III-IV according to the International Federation of Gynaecology and Obstetrics (FIGO), reflecting widespread intra-abdominal disease with a large number of metastatic tumor cells. OC cells in effusions probably represent a chemoresistant population rendering the disease untreatable and fatal (7,8).

Different cytologic biomarkers are used as adjuncts to morphologic examination to diagnose cancer cells in effusions (5). Studies focusing on molecules that promote the process of invasion and metastasis, as well as influence intracellular signalling pathways and/or act as transcription factors, have provided a better understanding of the biological events behind formation of malignant effusions (5,8); however, this knowledge is still far from complete. Although a growing number of investigations have defined optimal panels for routine cytologic diagnosis of carcinoma cells in effusions, only few studies focused on the molecular alterations and genetic mechanisms behind effusions (5,9,10). And yet, the identification of genetic mutations and genomic imbalances in tumor cells has become increasingly important in the management of different cancer types and also allows us to assess the cells' proneness to develop metastases (11,12).

We investigated the mutation status of the tumor suppressor gene *TP53*, the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), the protooncogenes of the Ras family-ki-ras2 kirsten rat sarcoma viral oncogene homolog (*KRAS*), Harvey rat sarcoma viral oncogene homolog (*HRAS*), the neuroblastoma RAS viral (V-Ras) oncogene homolog (*NRAS*)-and the v-raf murine sarcoma viral oncogene homolog (*BRAF*) in a series of 103 ovarian effusions. Furthermore, we performed array comparative genomic hybridization (aCGH) to characterize the genomic imbalances incurred by the cells of 20 effusions from HGSC, of which ten tumors showed *TP53* mutations whereas the remaining ten had wild-type *TP53*.

Materials and methods

Tumor material. The material consisted of 103 effusions from ovarian cancers, including 84 HGSC, 10 LGSC, two CCC, one EC, and six CS. All patients were treated at The Norwegian Radium Hospital between 2000 and 2015. The diagnoses were reached using a combination of cytological, morphological, and immunohistochemistry (IHC) investigations according to World Health Organization (WHO) 2014 guidelines (3). The study was approved by the Regional Committee for Medical and Health Research Ethics (REK, project number S-04300; http://helseforskning.etikkom.no), the government-appointed committee responsible for overseeing medical ethics in the South-East region of Norway. Informed consent, including consent for publication, was obtained according to national and institutional guidelines. An overview of the cohort used and the clinical and pathological data are given in Table I.

Molecular analyses. DNA was extracted using the Maxwell 16 extractor (Promega) and Maxwell 16 Cell DNA Purification kit (Promega) according to the manufacturer's recommendations. The concentration was measured using QIAxel (Qiagen).

Mutational analysis of *TP53*, *PIK3CA*, *KRAS*, *HRAS*, and *NRAS* was performed according to previously described protocols, using M13-linked PCR primers designed to flank and amplify targeted sequences (13,14). The primer combinations BRAF-F1 (5'TGCTTGCTCTGATAGGAAAATGAG ATCT3') and BRAF-R1 (5'ATCTCAGGGCCAAAAATT TAATCAGTG 3') were used to detect the mutation status of *BRAF*. The thermal cycling for *BRAF* included an initial step at 95˚C for 10 min followed by 35 cycles at 96˚C for 3 sec, 58˚C for 15 sec, 30 sec at 68˚C, and a final step at 72˚C for 2 min. Direct sequencing was performed using a 3500 Genetic Analyzer (Applied Biosystems).

The genes were selected based on the information reported in the COSMIC database (Catalogue of Somatic Mutations in Cancer, at https://cancer.sanger.ac.uk/cosmic) (15). According to COSMIC, there is no information on mutations in effusions; however, it contains data on the most frequently mutated genes in ovarian carcinoma. Since *KRAS* was in the top list, we decided to investigate also the other member genes of the RAS and RAF families, i.e., *HRAS*, *NRAS* and *BRAF*.

The BLAST (http://blast.ncbi.nlm.nih.gov/blast.cgi) and BLAT (http://genome.ucsc.edu/cgi-bin/hgblat) programs were used for computer analysis of sequence data. The reference sequences used for *TP53* was NM_000546.5.

Table I. Clinicopathologic parameters of the 103 ovarian effusions investigated.

Parameter	Distribution (n)
Histology	
HGSC	84
CS	6
LGSC	10
CCC	2
EC	1
Age	
≤60	42
>60	61
FIGO stage	
I	1
II	1
III	68
IV	33
Residual disease	
0 cm	23
≤1 cm	32
>1 cm	25
N/A	23
Chemoresponse after primary treatment[a]	
CR	53
PR	32
SD	7
PD	1
N/A	10

[a]N/A, Not available (missing data or disease response after chemotherapy could not be evaluated because of normalized CA 125 after primary surgery or missing CA 125 information and no residual tumor). HGSC, high-grade serous carcinoma; CS, carcinosarcoma; LGSC, low-grade serous carcinoma; CCC, clear cell carcinoma; EC, endometrioid carcinoma; N/A, not available; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

The difference between mutation and polymorphism was evaluated by the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org/variant/11-534242-A-G).

Whole genome investigation by means of aCGH was performed using the CytoSure Consortium Cancer + SNP arrays (Oxford Gene Technology) according the manufacturers' recommendation. Data were analysed using Agilent Feature Extraction Software (version 10.7.3.1) and CytoSure Interpret Software (version 4.9.40, Oxford Gene Technology). The genomic imbalances were identified using the Circular Binary Segmentation (CBS) algorithm and adding a custom-made aberration filter defining a copy number aberration (CNA) as a region with minimum five probes gained/lost (16). Annotations are based on human reference sequence GRCh37/hg19.

Twenty samples were selected for aCGH investigation, ten bearing *TP53* mutation in their genome and ten wild-type. The

Table II. Mutation status of *TP53*.

Case	Histology	*TP53*
1	HGSC	c.437G>A; p.W146*; COSM43609
2	HGSC	c.584T>C; p.I195T; COSM11089
3	HGSC	c.273G>A; p.W91*COSM44492
4	HGSC	
5	HGSC	c.916C>T; p.R306*; COSM10663
6	HGSC	
7[a]	HGSC	c.826_830delCCTGT
8	HGSC	c.818G>A; p.R273H; COSM10660
9	HGSC	c.797G>A; p.G266E; COSM10867
10	HGSC	
11	HGSC	c.488A>G; p.Y163C; COSM10808
12	HGSC	c.524G>A; p.R175H; COSM10648
13	HGSC	c.844C>T; p.R282W; COSM10704
14	HGSC	c.574C>T; p.Q192*; COSM10733
15	HGSC	c.527G>T; p.C176F; COSM10645
16	HGSC	c.469G>T; p.V157F; COSM10670
17	HGSC	c.527G>A; p.C176Y; COSM10687
18	HGSC	
19	HGSC	c.754del; p. L252fs*93; COSM45215
20	HGSC	c.403del; p.C135fs*35; COSM44670
21	HGSC	
22	HGSC	c.394A>T; p.K132*; COSM44641
23	HGSC	c.832C>G; p.P278A; COSM10814
24	HGSC	c.814G>A; p.V272M; COSM10891
25	HGSC	c.394A>G; p.K132E; COSM10813
26[a]	HGSC	c.475_476GC>TT
27	HGSC	
28	HGSC	c.797G>A; p.G266E; COSM10867
29	HGSC	c.108G>A; p.P36P; COSM6474191
		c.737T>A; p.M246K; COSM44103
30	HGSC	c.742C>T; p.R248W; COSM10656
31	HGSC	
32	HGSC	c.488A>G; p.Y163C; COSM10808
33	HGSC	c.836G>A; p.G279E; COSM43714
34	HGSC	
35	HGSC	c.818G>A; p.R273H; COSM10660
36	HGSC	
37	HGSC	
38	HGSC	
39	HGSC	c.524G>A; p.R175H; COSM10648
40	HGSC	
41	HGSC	c.711G>A; p.M237I; COSM10834
42	HGSC	
43	HGSC	c.166G>T; p.E56*; COSM12168
44	HGSC	c.524G>A; p.R175H; COSM10648
45	HGSC	
46	HGSC	
47	HGSC	
48	HGSC	
49	HGSC	
50	HGSC	
51	HGSC	
52	HGSC	c.434T>C; p.L145P; COSM43899
53	HGSC	

Table II. Continued.

Case	Histology	*TP53*
54	HGSC	
55	HGSC	c.475G>C; Pa159P; COSM43836
56	HGSC	
57	HGSC	
58	HGSC	
59	HGSC	
60	HGSC	c.844C>T; p.R282W; COSM10704
61	HGSC	c.646G>A; p.V216M; COSM10667
62	HGSC	c.832 C>T; p.P278S; COSM10939
63	HGSC	
64	HGSC	
65	HGSC	
66	HGSC	
67	HGSC	
68	HGSC	
69	HGSC	
70	HGSC	
71	HGSC	c.527G>T; p.C176F; COSM10645
72	HGSC	
73	HGSC	
74	HGSC	
75	HGSC	c.578A>G; p.H193R; COSM10742
76	HGSC	
77	HGSC	
78	HGSC	
79	HGSC	
80	HGSC	
81	HGSC	c.796G>A; p.G266R; COSM10794
82	HGSC	c.844C>T; p.R282W; COSM10704
83	HGSC	
84	HGSC	
85	LGSC	c.750del; p. I251fs*94; COSM44064
86	LGSC	
87	LGSC	c.714T>A; p.C238*; COSM45677
88	LGSC	
89	LGSC	
90	LGSC	
91	LGSC	
92	LGSC	
93	LGSC	
94	LGSC	
95	CCC	
96	CCC	
97	EC	c.1024C>T; p.R342*; COSM11073
98	CS	c.796G>A; p.G266R; COSM10794
99	CS	
100	CS	
101	CS	
102	CS	
103	CS	

[a]Novel mutation site; *, stop codon; HGSC, high-grade serous carcinoma; CS, carcinosarcoma; LGSC, low-grade serous carcinoma; CCC, clear cell carcinoma; EC, endometrioid carcinoma.

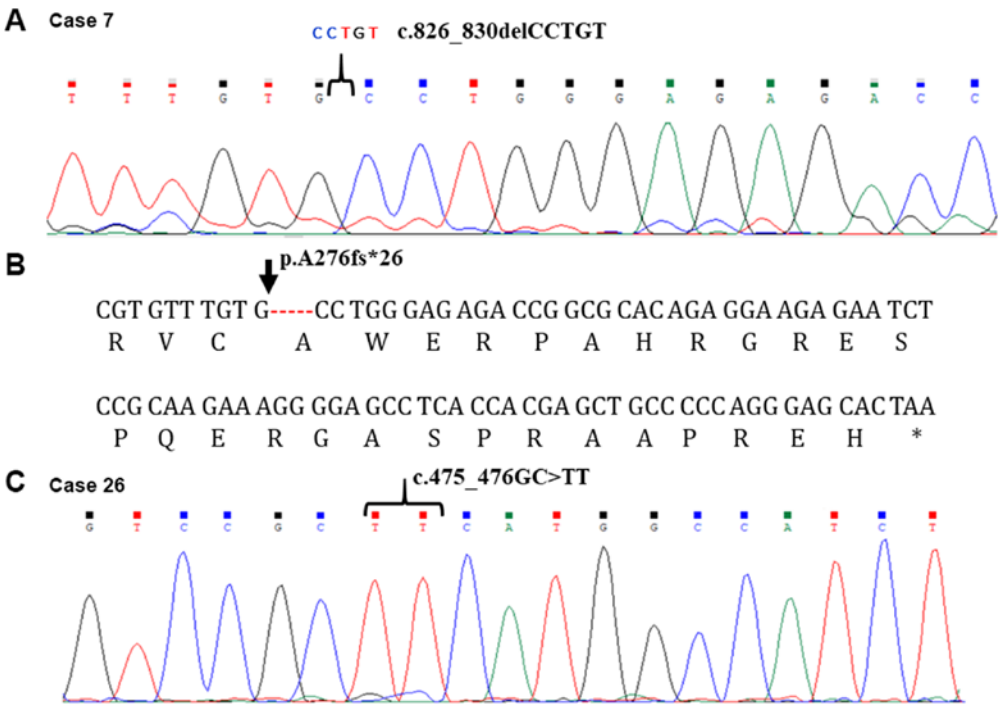

Figure 1. Novel site mutations for *TP53* (A) Partial sequence chromatogram of case 7 showing delCCTGT; (B) open reading frame of case 7; (C) partial sequence chromatogram of case 26 showing the substitution GC>TT.

average copy number alteration (ANCA) index was calculated as the total number of aberrations divided by the samples number between the two groups (17). The statistical analysis was performed using the Mann-Whitney U test.

Results

All effusions analyzed for *TP53*, *PIK3CA*, *KRAS*, *HRAS*, *NRAS*, and *BRAF* mutation status gave informative results. *TP53* was found mutated in 41 out of 84 HGSC (49%), in two out of 10 LGSC (20%), in the only case of EC examined, and in one out of six CS. A detailed overview of the *TP53* findings is shown in Table II. Two novel mutation sites were identified for *TP53*: c.826_830delCCTGT in case 7 and c.475-476GC>TT in case 26 (Fig. 1). *PIK3CA* mutations were found in four HGSC of 103, in which a c.1634A>C (cases 2, 56, and 58) and a c.3155C>T mutation (case 79) were seen. We identified the c.34G>T and c.183A>C *KRAS* mutations in two of 103 specimens (cases 10, a HGSC, and 85, an LGSC, respectively). The *HRAS* mutation c.173C>T was also detected in two tumors (2%; cases 16 and 23), both of them HGSC. Finally, we identified an *HRAS* polymorphism, c.81T>C, in 38 effusions (37.5%) of all histotypes. None of the tumors showed a mutated sequence for *NRAS* or *BRAF*.

aCGH analysis for genomic imbalances was performed on 20 effusions from patients with HGSC, comparing 10 tumors bearing *TP53* mutations (cases 1, 3, 5, 7, 8, 13, 14, 15, 19, and 32) and 10 which had a wild-type *TP53* sequence (cases 18, 27, 31, 36, 37, 38, 42, 45, 47, and 48). Overall, the aCGH analysis revealed highly imbalanced genomes in all tumors analysed with many gains and/or losses (Table SI). The most frequent gains were scored at 8q24.3, 20q13.2, and 20q13.31 (70%) whereas the most frequent losses were scored at 4q25 and 4q26 (75%) (Fig. 2). Amplifications mostly involved chromosomal band 19q11 followed by the segment 3q22q29. The two subgroups of effusions, i.e., with and without *TP53* mutation, were both very complex and similar with regard to imbalances. The ANCA index calculated for tumors (18) with *TP53* mutation was 83.2 but 66.3 for tumors with wild-type *TP53* (P=0.14).

Discussion

Molecular profiles of different tumor types have helped manage cancer patients with regard to diagnosis, prognosis, and lately also choice of treatment (19). A similar molecular characterization of effusions from ovarian cancer might highlight the mechanisms behind development of metastasis and possibly, further down the road, help decide among different personalized therapies (5). Since the number of studies focusing on molecular analysis of ovarian cancers at such advanced stage that effusions have already developed, is low, and since chemoresistance is one of the main characteristics of these malignancies, we aimed to add to the existing knowledge by performing mutation analyses of selected genes as well as determining copy number profiles of two groups of patients, those whose tumors did or did not have *TP53* mutations.

The tumor suppressor gene *TP53* has been found mutated in many different malignancies (20), including those arising in the ovaries, at a frequency of 66% in the most aggressive serous carcinomas (21). The rate of *TP53* mutation detected in our series was 46% for effusions from HGSC and LGSC. The seeming discrepancy between the frequencies recorded in the present series and in the literature could be due to methodological limitations, see below. In HGSC, we identified

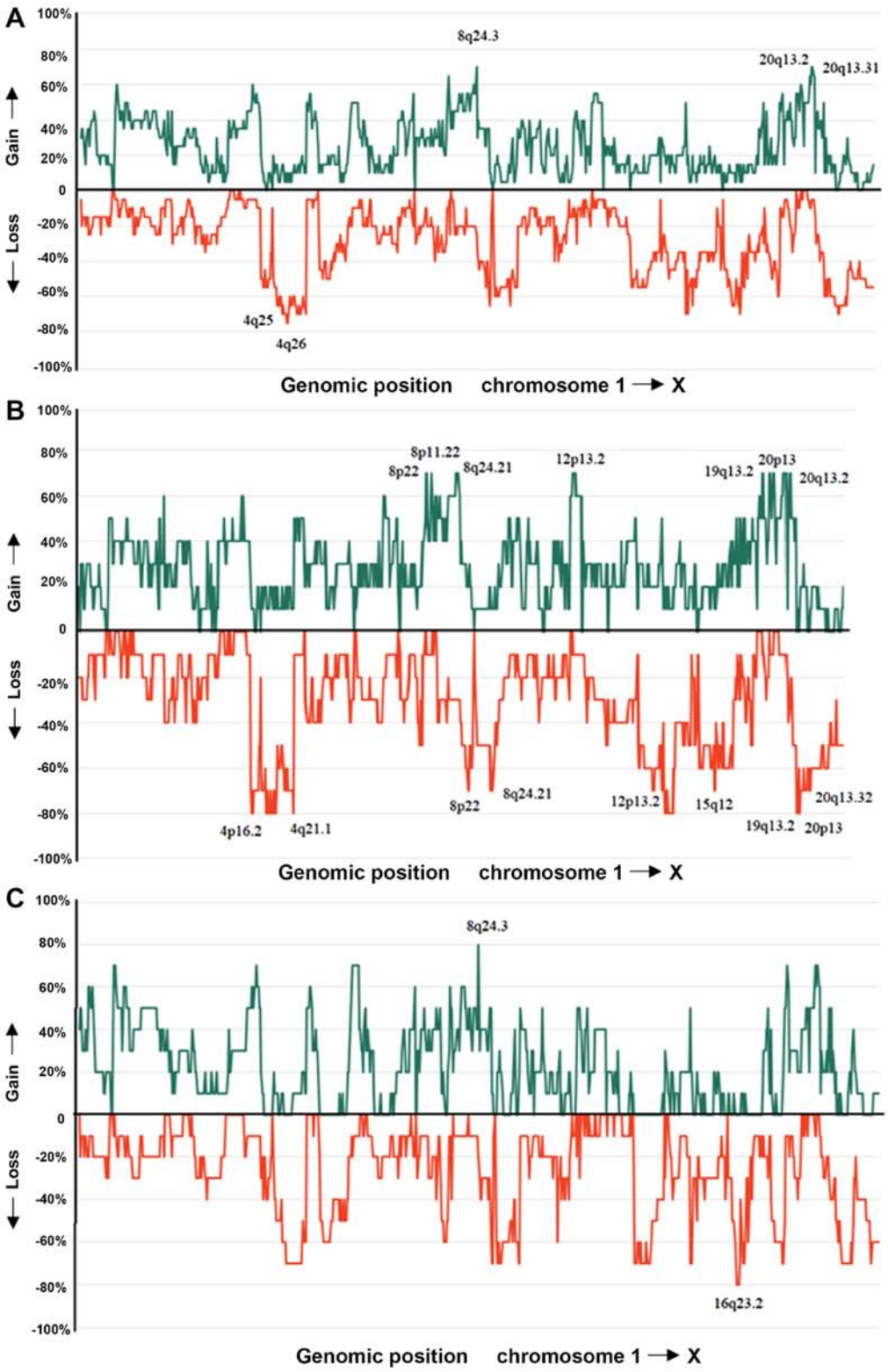

Figure 2. Profiles of imbalances detected by aCGH. (A) Gains and losses detected in effusion cells in patients with HGSC whose tumors were either *TP53* mutated or wild-type; (B) Genomic gains and losses in *TP53* mutated HGSC effusions; (C) Genomic gains and losses in *TP53* wild-type HGSC effusions. aCGH, array Comparative Genomic Hybridization; HGSC, high-grade serous carcinoma.

two novel sites for *TP53* mutation: A deletion of the CCTGT sequence was found in position c.826_830 of case 7 (stage III tumor), whereas a substitution GC>TT in position 475_476GC was identified in case 26 (stage IV tumor). The c.826_830del CCTGT is an out-of-frame change resulting in a frameshift of 26 amino acids (aa) (p. A276fs*26) (Fig. 1) after which a stop codon occurs. The predicted protein would consist of 156 aa. The substitution c.475_476GC>TT results in a change from alanine (A) to phenylalanine (F) (p.A159F). The mutation is at present of unknown pathogenicity in ovarian cancer. However, other mutations on c.475 have been reported as pathogenic in the COSMIC database, e.g., in tumors of the lung and liver

(https://cancer.sanger.ac.uk/cosmic). The impact of the new mutation sites in relation to different clinical parameters awaits further studies, ideally of larger series of patients. The two patients here examined had received upfront surgery and standard chemotherapy; case 7 showed a residual disease of 6 cm whereas case 26 had no residual disease at primary operation. Furthermore, both cases showed relatively long survival: Case 7 had 13 months progression-free survival (PFS) and overall survival (OS) of 81 months, whereas case 26 had PFS of 27 months and OS of 45 months.

PIK3CA belongs to the family of genes encoding phosphatidylinositol 3-kinases (PI3Ks). It is activated through the PI3K/AKT signalling pathway in 70% of ovarian cancers, promoting cellular growth, proliferation, and cell survival (22). Somatic mutations of this gene have been detected in different cancer types (23). In ovarian cancer, it occurs in 30% of all tumors, but reaches 45% in EC and CCC (24). We found *PIK3CA* mutated in 4% of the HGSC effusions examined, which is in line with what is reported in the COSMIC database. Unfortunately, the number of EC and CCC samples was too low to allow statistical conclusions. A number of clinical studies have focused on the PI3K/AKT/mTOR signaling pathway as a therapeutic target for patients with ovarian cancer (25,26); the identification of patients carrying *PIK3CA* mutation may therefore be important for the choice of therapy. Important to note in this regard is the fact that also other genes of the PI3K/AKT/mTOR signaling pathway should be investigated for their mutation status as they, too, may be involved pathogenetically (26).

KRAS and *HRAS* are principal members of the RAS family and have frequently been implicated in the development of different types of tumors (27). In ovarian carcinomas, the incidence of *KRAS* point mutations was found to be 13% (21). Previous studies have demonstrated an association between *KRAS* mutations and well-differentiated, clinically less advanced cancers (28,29). *KRAS* mutation was in ovarian serous carcinoma found more frequently in LGSC than in HGSC (30-32).

HRAS mutations are rare in ovarian tumors (33,34). We found an *HRAS* mutation in only two HGSC: However, our study showed presence of the 81T>C polymorphism in the coding region of *HRAS* in 38 out of 103 tumors (37%) of all histotypes. The Genome Aggregation Database, gnomAD, reports that SNP 81T>C is a polymorphism seen in 30% of the normal population. Both tumors with *HRAS* mutation also showed *TP53* mutation. In each case, one can hypothesize a scenario in which the mutations represent a primary and a secondary event either in the same cell or in different cells/clones.

Information on effusions from CS arising in the female genital tract is limited to data generated by immunohistochemical techniques (35). This is the first time that mutation analyses have been performed on such metastatic cells. It seems, however, that the genes investigated in the present study are not relevant in cells from effusions since we found only one CS with *TP53* mutation.

The mutation rates for the analysed genes in the present study differ slightly from those reported in the literature, something that may be attributable to the molecular methods applied. We used PCR followed by Sanger sequencing. It is known that Sanger sequencing cannot detect mutation if the level of abnormal cells is below 15% (36), whereas next generation sequencing (NGS) or exome sequencing, used in most published studies (37), is more sensitive, i.e., has a higher resolution level. NGS, on the other hand, cannot discriminate between a 'real' mutation and a polymorphism. Taking into account these two factors, one would indeed expect higher mutation rates to be detected by NGS compared to Sanger sequencing, as was observed.

aCGH data showed highly imbalanced genomes both in tumors with mutated and wild-type *TP53*. The genomic regions involved are in agreement with the results of previous studies where primary OC were investigated (38). The ANCA index detected in the *TP53* mutated subgroup was 83.2 whereas it was 66.3 in the subgroup with wild-type *TP53*. The difference between the two groups was not found statistically significant using the Mann-Whitney U test.

The origin of ovarian carcinomas has lately been debated but, according to the latest WHO classification, the majority of HGSC are thought to originate in the tubes whereupon metastatic spreading occurs to the ovaries (39,40). In the light of this concept, it is not surprising that ovarian carcinomas show the same imbalances as do ovarian cancer cells found in effusions, since both represent late evolutionary stages in carcinoma development.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MB performed molecular experiments and wrote the manuscript. IP participated in performing molecular experiments and interpretation of data. IK participated in performing data analysis. BD provided clinical data and specimens. SH assisted with writing of the article and experimental design. FM designed the study and supervised the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The ethical approval was granted by the Regional Committee for Medical and Health Research Ethics (REK; http://helseforskning.etikkom.no); for further information, please see this website: http://www.eurecnet.org/information/norway.html.

Patient consent for publication

Consent for publication of data was provided by all patients.

Competing interests

The authors declare that they have no competing interests.

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