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Frequency of mutations and polymorphisms in borderline ovarian tumors of known cancer genes

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Borderline ovarian tumors represent an understudied subset of ovarian tumors. Most studies investigating aberrations in borderline tumors have focused on *KRAS/BRAF* mutations. In this study, we conducted an extensive analysis of mutations and single-nucleotide polymorphisms (SNPs) in borderline ovarian tumors. Using the Sequenom MassArray platform, we investigated 160 mutations/polymorphisms in 33 genes involved in cell signaling, apoptosis, angiogenesis, cell cycle regulation and cellular senescence. Of 52 tumors analyzed, 33 were serous, 18 mucinous and 1 endometrioid. *KRAS* c.35G > A p.Gly12Asp mutations were detected in eight tumors (six serous and two mucinous), *BRAF* V600E mutations in two serous tumors, and *PIK3CA* H1047Y and *PIK3CA* E542K mutations in a serous and an endometrioid BOT, respectively. *CTNNB1* mutation was detected in a serous tumor. Potentially functional polymorphisms were found in *vascular endothelial growth factor* (*VEGF*), *ABCB1*, *FGFR2* and *PHLPP2*. *VEGF* polymorphisms were the most common and detected at four loci. *PHLPP2* polymorphisms were more frequent in mucinous as compared with serous tumors ($P = 0.04$), with allelic imbalance in one case. This study represents the largest and most comprehensive analysis of mutations and functional SNPs in borderline ovarian tumors to date. At least 25% of borderline ovarian tumors harbor somatic mutations associated with potential response to targeted therapeutics.

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Borderline ovarian tumors account for 10% of all ovarian neoplasms predominantly affecting women in the reproductive age.[1] Borderline ovarian tumors are histologically a heterogeneous group of slow growing, noninvasive tumors, the majority (85%) of which present with stage I disease confined to the ovary. Overall, borderline ovarian tumors have a significantly better prognosis with over 95% 5-year overall survival compared with 30–40% 5-year overall survival rates for their invasive counterparts.[1,2] Approximately 1% of borderline ovarian tumors show progression to invasive epithelial cancer. Non-resectable recurrent disease is responsible for the majority of disease-related deaths and present similar problems to invasive ovarian cancer, such as bowel obstruction and drug resistance.[1]

Prognostic factors in patients with borderline ovarian tumors and features potentially associated with recurrent and/or progressive disease include tumor type, patient age, FIGO stage, invasive implants, microinvasion in the primary tumor and micropapillary architecture.[3–6] No single clinical or pathological feature or combination of features identify all adverse outcomes.[7] Treatment options

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for patients with persisting/progressive disease are limited,[8,9] with limited activity of currently available chemotherapeutic agents used in invasive ovarian cancers in the treatment of borderline ovarian tumors.[9,10] New systemic therapeutic agents are therefore urgently required.[1]

Although the clinical and pathological characteristics of borderline ovarian tumors are well described, the molecular aspects are poorly understood.[11,12] Borderline ovarian tumors have not been as extensively studied at a molecular level as invasive ovarian carcinomas, and knowledge of genetic abnormalities associated with borderline ovarian tumors is limited. Studies looking into gene mutations in borderline ovarian tumors have mainly been in comparison with invasive carcinoma and focused on *TP53*, *BRAF* and *KRAS*.[13–16] *TP53* mutations are not commonly associated with borderline ovarian tumors, in contrast to their high frequency in high-grade carcinoma.[15] Conversely, *KRAS* and *BRAF* mutations are both much more common in borderline ovarian tumors and low-grade serous carcinomas.[13,17]

In addition to *KRAS*, *BRAF* and *TP53*, a few studies have also investigated the frequency of *PIK3CA*,[18] *BRCA1*,[19] *EGFR*,[20] *CTNNB1*[21] and *PTEN*[21] mutations in borderline ovarian tumors in comparison with invasive ovarian carcinomas. Gene amplifications have also been studied in borderline ovarian tumors, including *ERBB2* (ref.22) and *AKT2*.[23]

In this study, we used the high-throughput Sequenom MassArray approach to investigate single-nucleotide mutations and polymorphisms in 33 genes in a cohort of borderline ovarian tumors to determine the frequency of genetic changes associated with borderline ovarian tumors (see Supplementary Appendix 1in the Supplemental Material).

Materials and methods

Tumor Samples

Frozen tissue from 52 borderline ovarian tumors was obtained from the Imperial College Healthcare NHS Trust Tissue Bank, Hammersmith Hospital. Ethics Committee approval for use of human tissue was obtained. Table 1 summarizes patients' age, tumor types and tumor stage.

Table 1 Patient and tumor characteristics

Age (median, years)	*50*
Tumor type	
Endometroid	1
Mucinous	18
Serous	33
FIGO stage	
IA	22
IC	16
IIA	2
IIC	1
IIIA	1
IIIB	3
IIIC	1
Unstaged	6

DNA Extraction

DNA was extracted from fresh snap frozen tissue. An H&E-stained section from the frozen tissue used for each specimen was examined to verify the content and the quality of the tissue analyzed. Briefly, tissue was homogenized in 180 μl RTL buffer (Qiagen) using a TissueLyser (Tissuelyser I, Retsch, Leeds, UK) at 15 Hz, for 20 s. Supernatant containing disrupted tissue was transferred to a 1.5-ml microfuge tube, 20 μl Proteinase K was added and the sample incubated at 56 °C overnight. DNA was then extracted using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocol.

Sequenom MassArray

The list of genes, mutations and polymorphisms assessed by Sequenom are presented in Supplementary Appendix 1, Supplemental Material. PCR and extension primers were designed using Assay Design (Sequenom). PCR-amplified DNA was cleaned using EXO-SAP (Sequenom), and primer was extended by IPLEX chemistry, desalted using Clean Resin (Sequenom) and spotted onto Spectrochip matrix chips using a nanodispenser (Samsung). Chips were run in duplicate on a Sequenom MassArray MALDI-TOF MassArray system. Sequenom Typer Software and visual inspection were used to interpret mass spectra. Reactions where >15% of the resultant mass ran in the mutant site in both reactions were scored as positive. Mutations and polymorphisms for a subset of samples and targets were confirmed by Sanger sequencing and pyrosequencing, respectively.

Pyrosequencing

PHLPP2 polymorphisms and allelic imbalance in tumors was assessed using pyrosequencing of genomic DNA. The primers for *PHLPP2* amplification and sequencing were: 5′-AAACAAAGCATTGT GGGAACACT-3′ (forward), 5′-biotin-AAACTACCAT CGCCCCTACATT-3′ (reverse) and 5′-CTAAGAAGC TGTGCACAT-3′(sequencing). Initial PCR was performed using Jumpstart Taq (Sigma), 60 °C annealing, 2.5 mM MgCl, 200 nM primer and 10 ng genomic DNA. Pyrosequencing of PCR products was performed using PyroGold Reagent kit (Biotage, Uppsala, Sweden) according to the manufacturer's instructions. The individual genotypes of the rs61733127 (L1016S) polymorphism were estimated manually using the Pyro Q-CpG Software (Qiagen, UK)) with thresholds for TT (<10% C),

CT (40–60% C) and CC (>90% C). We used the quantitation of C *vs* T alleles in heterozygotes to identify tumors displaying loss of heterozygosity with a threshold of 10–40% C.

Sanger Sequencing for KRAS

Mutations in *KRAS* were verified using primers (forward: 5′-TTTGATAGTGTATTAACCTTATG-3′, reverse: 5′-GAGGTAAATCTTGTTTTAATA-3′), using 10 ng DNA, 200 nM primer, 2.5 mM MgCl and JumpStartTaq (Sigma) at 52 °C for 40 cycles. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Cycle conditions were 94 °C for 1 min followed by 30 cycles of 94 °C for 10 s, 55 °C for 15 s and 60 °C for 4 min. PCR products were cleaned by EDTA–ethanol precipitation, resuspended in HiDi formamide and run on a 3730 × l DNA Analyzer (Applied Biosystems Ltd). Base calling, quality assessment and assembly were carried out using the Phred, Phrap, Polyphred, Consed software suite. All potential sequence variants were verified by manual inspection of the chromatograms.

EGFR and PDGFRA Mutational Analysis

EGFR mutations in exons 19–21 and *PDGFRA* mutations in exons 12 and 18 were analyzed by capillary electrophoresis single-strand conformation analysis (CE-SSCA) using a 3130 × l genetic analyzer (Life Technologies, Warrington, UK) and non-denaturing polymer at three different temperatures. Any conformation changes were subjected to bidirectional Sanger sequencing.

Immunohistochemistry

The expression of beta-catenin was evaluated by immunohistochemistry using the avidin–biotin immunodetection complex method. Two-micron-thick sections from formalin-fixed, paraffin-embedded tissue were prepared, deparaffinised and rehydrated. Endogenous peroxidase was blocked by incubation in hydrogen peroxide. Antigen retrieval was performed by microwaving in 0.01 M citrate buffer (pH 6.0) at 750 W for 20 min. Nonspecific binding was blocked with normal goat serum for 10 min. Tissue sections were then incubated with primary antibody for beta-catenin (BD Biosciences, 1:500 dilution) at room temperature for 60 min. The sections were washed and then incubated with biotinylated goat anti-mouse immunoglobulin (Dako, 1:2000 dilution) for 30 min, followed by streptavidin peroxidase for 30 min. The slides were developed in DAB and followed by a hematoxylin counterstain. For each case, a section in which the primary antibody was replaced by phosphate-buffered saline was used as a negative control.

Statistical Analysis

The χ^2 test was used to test for the presence of associations between the different gene mutations and polymorphisms and the histopathological features of tumors. A *P*-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS (version 16.0, Chicago, IL, USA).

Results

Fifty-two borderline tumors were studied (Table 1 and Supplementary Appendix 2 in the Supplementary Material). The tumors included 33 serous (63%), 18 mucinous (35%) and 1 endometrioid tumor (2%). Six (18%) of the serous tumors had a micropapillary component, two (6%) showed microinvasion and seven (21%) were associated with noninvasive implants. Of the mucinous tumors, two (11%) showed microinvasion and four (22%) showed intramucosal carcinoma. Patients aged from 26 to 82 years (median 50 years), with a median follow-up period of 3 years (range 2–7 years). One patient with a mucinous borderline ovarian tumor treated with unilateral oophorectomy developed a cyst on the other ovary that was detected on a follow-up scan 18 months after initial surgery. The patient did not undergo surgical removal of the cyst, so the histological nature of the cyst is unknown to us. None of the patients with serous and endometrioid tumors or other patients with mucinous tumors developed recurrence or disease progression.

Borderline endometrioid tumors are very rare, and this was an unusual opportunity to study the status of a large set of genes in this tumor.

Gene Mutations

Mutations were detected in 6 (*KRAS*, *BRAF*, *PIK3CA*, *EGFR*, *PDGFRA* and *CTNNB1*) of the 33 genes studied using the Sequenom assay (Table 2, Supplementary Appendix 2 in the Supplementary Material). *KRAS* c.35G>A p.Gly12Asp mutations were detected in 8/52 tumors (15%), which included 6/33 serous (18%) and 2/18 mucinous (11%) tumors. *BRAF* mutations were detected in only 2/52 (4%) tumors, both of which were in serous tumors (2/33, 6%). *PIK3CA* mutations were found in 2/52 tumors (4%). *PIK3CA*_E542K (heterozygous mutation—het) was present in 1/33 (3%) serous tumor, and *PIK3CA*_H1047Y (het) was detected in the one borderline endometrioid tumor. *PDGFRA*_V824L (het) was detected in three tumors (6%). These included 2/33 serous (6%) and 1/18 (6%) mucinous tumors. CE-SSCA and Sanger sequencing analysis of these samples failed to confirm the *PDGFRA*_V824L mutation, instead, 2/3 cases showed a synonymous polymorphism very near to that genomic position (c.2472C>T; p.V834V). The third case showed

Table 2 Frequency of mutations

Mutation	Sequence	Genotype	Frequency (n = 52)
PIK3CA E542	1624G	GA	1/52 (2%) 1/33 serous (3%)
PIK3CA H1047Y		TC	1/52 (2%); 1/1 endometrioid (100%)
KRAS G12	35G		8/52 (15%)
		GA	4/33 serous (12%); 1/18 (6%) mucinous
		GT	2/33 (6%) serous; 1/18 (6%) mucinous
BRAF V600E	1799T	AT	2/52 (4%); 2/33 serous (6%)
CTNNB1 S37	110C	CG	1/52 (2%); 1/33 serous (3%)

heavily degraded DNA and a wild-type conformation, which can also result in false priming.

Sequenom also identified *EGFR* L858R mutations in five cases. However, these mutations were not validated on Sanger sequencing.

*CTNNB1*_S37C (het) mutation was detected in 1 of the 33 serous tumors (3%). Immunostaining of this sample showed nuclear localization of beta-catenin, a feature seen in the presence of *beta-catenin* mutations (Figure 1). This case was reviewed by two gynecological histopathologists and confirmed to be of the serous type.

Thirteen (25%) of 52 tumors had at least one mutation in the genes tested (Supplementary Appendix 2, Supplementary Material). Eleven of the 13 (85%) cases had a single mutation, while 2 cases (15%) had two mutations (*KRAS* and *CTNNB1* in one serous tumor and *KRAS*_G12_35G and *PIK3CA*_H1047Y in one endometrioid tumor). Table 3 summarizes the correlation between the presence of mutations and histopathological features of serous and mucinous tumors.

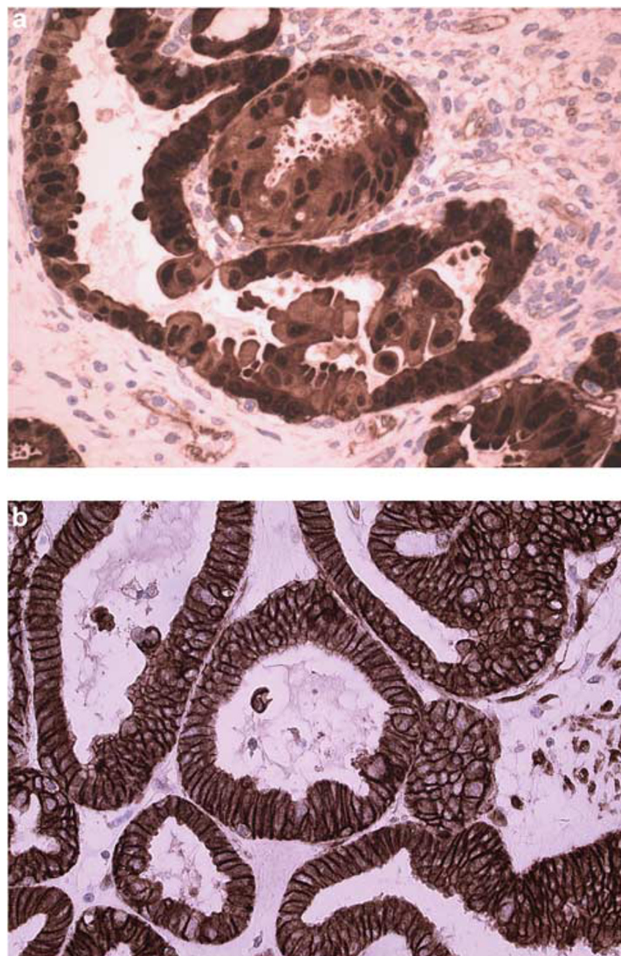

Figure 1 Expression of beta-catenin in a serous borderline ovarian tumor with *beta-catenin* mutation. In the serous borderline tumor with beta-catenin mutation there is cytoplasmic and notable nuclear localization with the absence of membranous staining (A: ×400). This is in contrast to distinct membranous localization and absence of nuclear localization in a serous borderline ovarian tumor with wild-type *beta-catenin* (B: ×100).

Gene Polymorphisms

Potential functional single-nucleotide gene polymorphisms (SNPs) were found in 4 of the 33 genes studied (Table 4, Supplementary Appendix 2, Supplemental Material). Only 4/52 (8%) of tumors featured SNPs in only one of the genes tested, whereas 48/52 (92%) tumors showed polymorphisms in two or more of the genes tested with the majority of genes being heterozygous (Table 4). The most common polymorphism observed was in *VEGF* in 50/52 borderline ovarian tumors (96%), with SNPs detected at four loci. There were two or more *VEGF* SNPs in 24/33 serous tumors, 1/1 endometrioid and 11/18 mucinous tumors. *ABCB1* polymorphisms were detected in 37/52 borderline ovarian tumors (71%), followed by *FGFR2* polymorphisms in 29/52 borderline ovarian tumors (56%).

Heterozygote *PHLPP2* polymorphisms were detected in 14/52 (27%) tumors. These included 5/33(15%) serous, 8/18 mucinous (44%) and one endometrioid borderline ovarian tumor. Tumors with *PHLPP2* polymorphisms included 2/4 (50%) mucinous tumors with intramucosal carcinoma and 1/6 (17%) serous tumor of the micropapillary type, and 1/2 (50%) serous tumors with microinvasion (50%).

Table 5 shows the correlation between polymorphisms and histopathological features of tumors. *PHLPP2* polymorphisms were more common in mucinous tumors compared with serous BOTs; 44% ($n = 8/18$) *vs* 15% ($n = 5/33$), χ^2 ($P = 0.04$).

Validation of Gene Mutations and Polymorphisms in Selected Genes

KRAS gene mutation status was assessed by direct Sanger sequencing in five cases, and confirmed the results of Sequenom MassArray in all cases. *PHLPP2* polymorphisms were confirmed by pyrosequencing in all 52 cases. In addition, relative allelic quantification demonstrated evidence of allelic

Table 3 Correlation between mutations and histopathological features of serous and mucinous tumors

	Noninvasive implants (S)		*Micropapillary component (S)*		*Microinvasion (S and M)*		*Intramucosal carcinoma (M)*	
	+	−	+	−	+	−	+	−
KRAS	1/7 (14%)	5/26 (19%)	0/6 (0%)	6/27 (22%)	0/2 S (0%) 0/2 M (0%)	6/31 S (19%) 2/16 M (13%)	0/4 (0%)	2/14 (14%)
BRAF	0/7 (0%)	2/26 (7%)	0/6 (0%)	2/27 (7%)	0/2 S (0%) 0/2 M (0%)	2/31 S (6%) 0/16 M (0%)	0/4 (0%)	0/14 (0%)
PIK3CA	0/7 (0%)	1/26 (4%)	0/6 (0%)	1/27 (4%)	0/2 S (0%) 0/2 M (0%)	1/31 S (3%) 0/16 M (0%)	0/4 (0%)	0/14 (0%)
β-Catenin	0/7 (0%)	1/26 (4%)	0/6 (0%)	1/27 (4%)	0/2 S (0%) 0/2 M (0%)	1/31 S (3%) 0/16 M (0%)	0/4 (0%)	0/14 (0%)

Abbreviations: S = serous; M = mucinous.

Table 4 Frequency of polymorphisms

Polymorphism	*PHLPP2_L1016S_T3047C*	*ABCB1_G2677TA*	*FGFR2_rs2981582_CT*	*VEGF_5_1154_GA_ref*	*VEGF_5_1498_CT*	*VEGF_5_2573_CA*	*VEGF_5_634_GC_ref*
Serous	5/33 (15%)* (CT)	23/33 (70%) (T: 8; GT: 15)	18/33 (55%) (T: 8; CT: 10)	13/33 (39%) (GA)	26/33 (79%) (T: 4; TC: 22)	7/33 (21%) (CA)	23/33 (70%) (C: 4; CG: 19)
Mucinous	8/18 (44%) *(CT)	13/18 (72%) (T: 4; GT: 9)	11/18 (61%) (T: 2; CT: 9)	9/18 (50%) (A: 1; GA: 8)	12/18 (67%) (T: 3; TC: 9)	1/18 (6%) (A)	9/18 (50%) (C: 2; CG:7)
Endometrioid	1/1 (100%) (CT)	1/1 (100%) (GT)	0/1 (0%)	0/1 (0%)	1/1 (100%) (TC)	0/1 (0%)	1/1 (100%) (CG)

Table 5 Correlation between polymorphisms and histopathological features of serous and mucinous tumors

	Noninvasive implants (S)		*Micropapillary component (S)*		*Microinvasion (S and M)*		*Intramucosal carcinoma (M)*	
	+	−	+	−	+	−	+	−
PHLPP2_L1016S_T3047C	0/7 (0%)	5/26 (19%)	2/6 (33%)	3/27 (11%)	1/2 S (50%) 0/2 M (0%)	4/31 S (13%) 8/16 M (50%)	2/4 (50%)	6/14 (43%)
ABCB1_G2677TA	4/7 (57%)	19/26 (73%)	5/6 (83%)	18/27 (67%)	1/2 S (50%) 1/2 M (50%)	22/31 S (71%) 12/16 M (75%)	1/4 (25%)	12/14 (86%)
FGFR2_rs2981582_CT	3/7 (43%)	15/26 (58%)	4/6 (66%)	13/27 (48%)	1/2 S (50%) 2/2 M (100%)	17/31 S (55%) 9/16 M (56%)	3/4 (75%)	8/14 (57%)
VEGF_5_1154_GA_ref	1/7 (14%)	12/26 (46%)	2/6 (33%)	11/27 (41%)	1/2 S (50%) 1/2 M (50%)	12/31 S (39%) 8/16 M (50%)	2/4 (50%)	7/14 (50%)
VEGF_5_1498_CT	6/7 (86%)	20/26 (77%)	5/6 (83%)	21/27 (78%)	2/2 S (100%) 2/2 M (100%)	24/31 S (77%) 10/16 M (62%)	3/4 (75%)	9/14 (64%)
VEGF_5_2573_CA	1/7 (14%)	6/26 (23%)	1/6 (17%)	6/27 (22%)	2/2 S **(100%) 0/2 M (0%)	5/31 S **(16%) 1/16 M (6.2%)	0/4 (0%)	1/14 (7%)
VEGF_5_634_GC_ref	5/7 (71%)	18/26	4/6 (66%)	19/27 (70%)	2/2 S (100%) 0/2 M	21/31 S 9/16 M	3/4 (75%)	6/14 (43%)

Abbreviations: S = serous; M = mucinous.

imbalance in one borderline ovarian tumor (Figure 2).

Discussion

We used the Sequenom MassArray technique to profile gene single-nucleotide mutations and polymorphisms in borderline ovarian tumors. We and others have previously demonstrated that the sensitivity of mass spectrometric methods exceeds that of traditional Sanger sequencing where the aberration must be present in ~20% of the DNA present and is highly concordant with Sanger sequencing, pyrosequencing and allele-specific PCR.[24,25] Fifty-two tumors, representing the largest set of borderline ovarian tumors analyzed for mutational status, were studied for changes in 33 genes known to be involved in tumor pathology with the majority being potential targets, including genes in the *RAS-RAF-MEK* and *RTK-phosphatidylinositol 3-kinase (PI3K)-AKT* pathways.

Our study of borderline ovarian tumors shows that somatic mutations occur predominately in a subset of the genes studied. Overall, the frequency and pattern of mutations is consistent with borderline ovarian tumors showing more similarity and being potential precursors of Type I ovarian

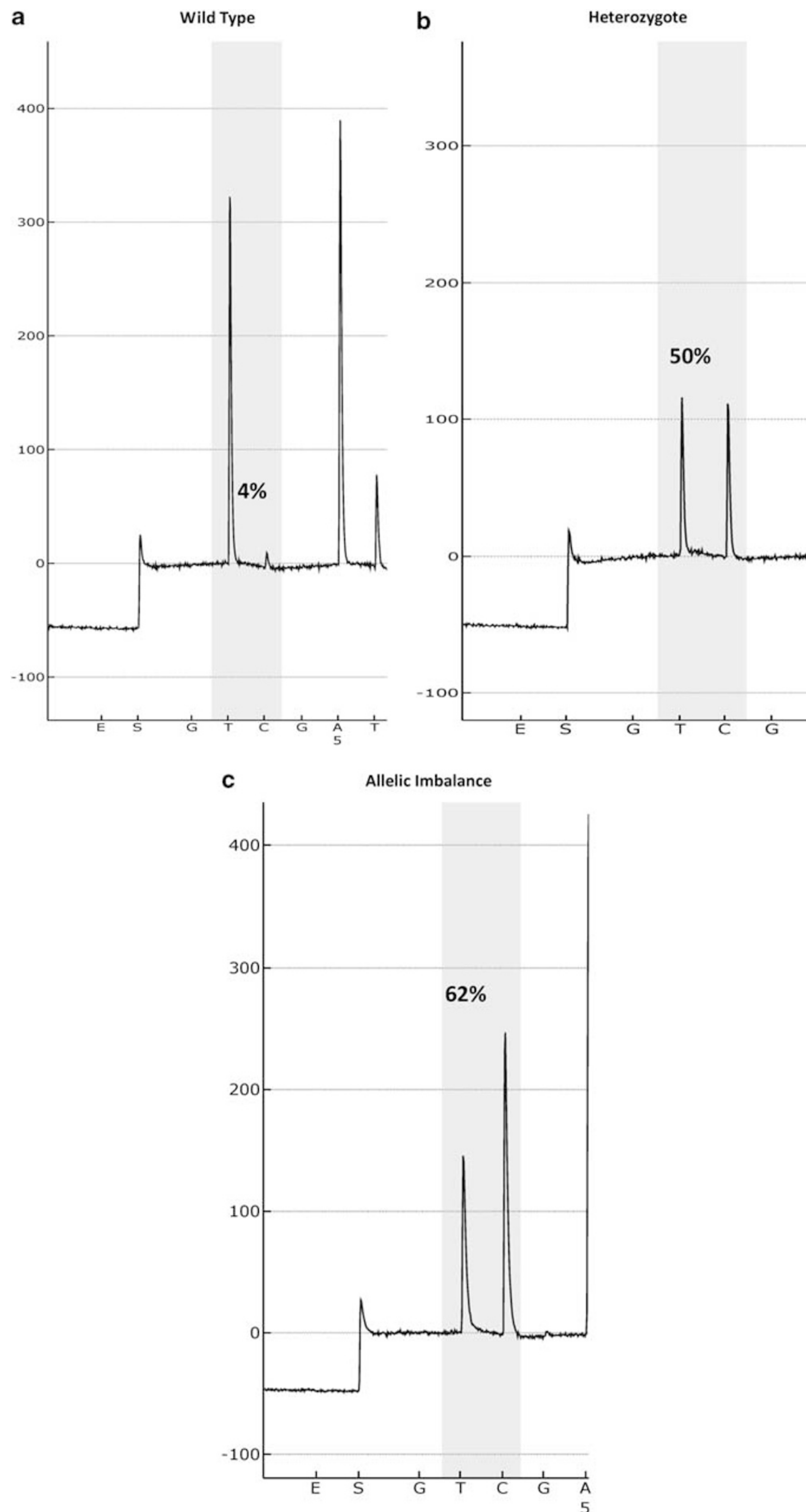

Figure 2 Pyrosequencing results for *PHLPP2* polymorphisms. (**a**) Normal female genomic DNA homozygous for *PHLPP2* major allele, (**b**) borderline ovarian tumor heterozygous for major and minor alleles and (**c**) borderline ovarian tumor with allelic imbalance for the minor allele.

carcinoma. Jones *et al* comprehensively analyzed somatic mutations in low-grade serous carcinomas, by exome sequencing, and showed that the genes showing the most frequent mutations were *BRAF* and *KRAS*, occurring in 38 and 19% of low-grade tumors, respectively, and a single case showed a *PIK3CA* mutation. Their mutational analysis demonstrates that point mutations are much less common in low-grade serous tumors of the ovary.[17] In agreement with previous reports, in our study *KRAS* and *BRAF* mutations were the most common mutations detected.[13,26–30] Mutations in decreasing order of frequency were identified in *KRAS, BRAF, PIK3CA* and *CTNNB1*. Mutations in these genes are likely to perturb several signaling cascades as well as signaling networks involved in cell proliferation, survival and motility. The most frequent polymorphisms were found in the *VEGF* gene with four SNPs, followed by *ABCB1, FGFR2* and *PHLPP2*. Allelic imbalance in favor of the minor allele of *PHLPP2* was shown in one tumor suggesting selection of the minor allele may have a role in tumor development and progression.

Although the frequency of *RAS/RAF* mutations in our cohort is relatively less than that in previous reports,[16,31] the majority of detected mutations and polymorphisms seemed to focus on the *RAS/RAF* and *PI3K/AKT* pathway and upstream growth factors and growth factor receptors. The *Ras/Raf/MEK/ERK* and *PI3K/PTEN/AKT* signaling cascades interact and have critical roles in the transmission of signals from growth factor receptors to regulate gene expression and prevent apoptosis. These signaling and anti-apoptotic pathways can have different effects on growth, prevention of apoptosis and induction of drug resistance in cells of various lineages. Components of these pathways or upstream receptors are mutated or aberrantly expressed in human cancer and subjects of active drug development programs.[32]

One serous tumor had concurrent *CTNNB1* and *KRAS* mutations, and an endometrioid borderline ovarian tumor *PIK3CA* and *KRAS* mutations. The finding of concordant mutations, despite the overall low frequency of mutations, suggests that additional genetic abnormalities are selected in the presence of mutations in *KRAS* and *BRAF* during the pathogenesis of borderline ovarian tumors. The presence of co-ordinate mutations suggests that alternative functions of the two genes are selected or that an aberration in a single node in the pathway does not engender sufficient pathway activation for tumor initiation or progression. *CTNNB1* mutations have been reported previously in borderline ovarian tumors, exclusively in endometrioid but not serous borderline ovarian tumors as in the case here.

Deregulated signaling via the PI3K pathway is common in many cancer lineages. For example, *PIK3CA* is the most commonly mutated oncogene in uterine endometrioid carcinoma (UEC)[33] and breast carcinomas.[34] Mutations occur predominately in exon 20 (kinase domain) and exon 9 (helical domain) in breast cancer[35–37] but in other sites in different lineages such as UEC. The frequency of PIK3CA hotspot mutations throughout the coding region of 3% is similar to the previously reported rate of 5% in borderline ovarian tumors.[18] In endometrial carcinoma, *PIK3CA* mutations occur more frequently in *KRAS*-mutant samples (7/18, 39%; $P = 0.06$) than in *KRAS* wild-type (17/90, 19%) tumors.[38] Consistent with these results, the only case of endometrioid borderline ovarian tumor harbored both *KRAS* and *PIK3CA* mutations. In contrast, and in keeping with published studies, *AKT* mutations were not identified in borderline ovarian tumors.[23]

The value of SNPs, the most common form of genetic variation, as biomarkers in cancer for risk and prognosis is well established. There may be quantitative variation of transcript levels associated with distinct alleles or haplotypes found in promoters and coding regions of genes. These changes in expression owing to allelic variation are often associated with additional genomic or transcript modifications, such as DNA methylation or RNA editing. The Sequenom MassArray platform is a rapid, high-throughput platform that has been extensively used for SNP detection.[39,40]

The physiological role of VEGF in angiogenesis, and the activity of anti-VEGF agents, such as bevacizumab, in ovarian cancer, makes it an important target for evaluation in genetic association studies.[41] Ovarian cancer patients with the *VEGF* C + 936T polymorphism C/T genotype have a longer median PFS of 11.8 months, compared with those with the C/C and T/T genotype, with median PFS of 5.5 and 3.2 months, respectively.[42] In our study, *VEGF* polymorphisms were the most common genetic variation detected, being present in 96% of tumors. Polymorphisms were found in four loci of the gene and 69% of the tumors had polymorphisms in two or more loci.

In this study, we detected polymorphisms and allelic imbalance in one of the members of the *PHLPP* gene family. The two members of this recently discovered family, *PHLPP1* and *PHLPP2*, control the degree of agonist-evoked signaling by Akt and the cellular levels of PKC.[43,44] Brognard *et al* identified a T-C SNP at position 3047 of *PHLPP2*, with a population frequency of 30%, which results in an amino-acid change from Leucine to Serine at codon 1016 in the PP2C phosphatase domain with a reduction in phosphatase activity, thus driving constitutive phosphorylation of Akt.[45]

In this study, we detected this *PHLPP2* polymorphism in 27% of tumors with a significant correlation with mucinous tumors as compared with serous. Pyrosequencing technology provides qualitative sequencing data simultaneously with quantitative allele dose information (allele quantification). Using pyrosequencing, we confirmed the presence of the *PHLPP2* polymorphisms detected on the

Sequenom platform, with loss of heterozygosity in one case. The association between *PHLPP2* polymorphism and mucinous borderline ovarian tumors to our knowledge has not previously been reported, and may imply a role for the activation of AKT/PKC pathways in the genesis of this phenotype.

*PDGFRA*_V824L (het) was detected in three tumors (6%). CE-SSCA and Sanger sequencing analysis of these samples failed to confirm the *PDGFRA*_V824L mutation, instead, 2/3 cases showed a synonymous polymorphism very near to that genomic position (c.2472C>T; p.V834V). In most primer designs, care in taken to avoid annealing positions that overlap known polymorphic sites; however, if secondary structure interferes with primer design on the opposite strand, this is not always possible. Sequenom also identified *EGFR* L858R mutations in five cases. However, these mutations were not validated on Sanger sequencing. The discordance can be owing to the increased sensitivity of the mass spec-based sequencing; these mutations were detected at an allelic frequency of 15%, which is below the detection level of Sanger sequencing.

Highly accurate MALDI-TOF-based detection provides unparalleled specificity and sensitivity for studies of genetic variation, including somatic mutation detection in heterogeneous samples. The limitations of this platform is that only 'hotspot' mutations are detectable. Although the Sequenom MassArray system is highly sensitive and accurate, before use in clinical practice, all mutations should be confirmed using an approved CLIA or equivalent assay in a laboratory medicine facility. Indeed, the *EGFR* and *PDGFRA* mutations detected by MALDI-TOF could not be confirmed by a validated clinical method based on CE-SSCA and Sanger sequencing. In the case of *PDGFRA* mutations, this discordance is likely due to the presence of a known polymorphism in the area adjacent to the mutation hotspot for which the test is designed in two out of three cases. Although our study was carried out on frozen tissue, these mutations and polymorphisms can in principle be detected on analyzing DNA extracted from formalin-fixed paraffin-embedded tissue, and hence can be used in routine practice. However, the degraded nature of some DNA samples derived from formalin-fixed paraffin-embedded tissue can cause artefacts in the analysis.

In summary, this study expands the repertoire of mutations and polymorphisms implicated in the pathogenesis of borderline ovarian tumors. The genes and pathways associated with these mutations and polymorphisms are clinically important with active drug development programs, as the trials using MEK inhibitors in low-grade serous carcinoma offer the opportunity for the implementation of similar targeted therapy in borderline ovarian tumors, an area of unmet clinical need.[46] A larger sample size linked to clinical trials is required to establish that a gene mutation has a significant impact for prediction of response to therapy or prognosis. Future studies to determine if the presence of specific mutations predicts antitumor activity of targeted biological agents in borderline ovarian tumors are warranted.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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