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Mutational analysis of *BRAF* and *KRAS* in ovarian serous borderline (atypical proliferative) tumours and associated peritoneal implants

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Abstract

There is debate as to whether peritoneal implants associated with serous borderline tumours/atypical proliferative serous tumours (SBT/APSTs) of the ovary are derived from the primary ovarian tumour or arise independently in the peritoneum. We analysed 57 SBT/APSTs from 45 patients with advanced-stage disease identified from a nation-wide tumour registry in Denmark. Mutational analysis for hotspots in *KRAS* and *BRAF* was successful in 55 APSTs and demonstrated *KRAS* mutations in 34 (61.8%) and *BRAF* mutations in eight (14.5%). Mutational analysis was successful in 56 peritoneal implants and revealed *KRAS* mutations in 34 (60.7%) and *BRAF* mutations in seven (12.5%). Mutational analysis could not be performed in two primary tumours and in nine implants, either because DNA amplification failed or because there was insufficient tissue for mutational analysis. For these specimens we performed VE1 immunohistochemistry, which was shown to be a specific and sensitive surrogate marker for a V600E *BRAF* mutation. VE1 staining was positive in one of two APSTs and seven of nine implants. Thus, among 63 implants for which mutation status was known (either by direct mutational analysis or by VE1 immunohistochemistry), 34 (53.9%) had *KRAS* mutations and 14 (22%) had *BRAF* mutations, of which identical *KRAS* mutations were found in 34 (91%) of 37

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Author contributions

LA performed experiments; FZ, RJK, I-MS and SKK helped in writing the manuscript and preparing illustrations and tables; CGH, RV, JJ and RJK helped in organizing cases and reviewing slides to confirm diagnosis; and LC helped with statistical analysis.

SBT/APST–implant pairs and identical *BRAF* mutations in 14 (100%) of 14 SBT/APST–implant pairs. Wild-type *KRAS* and *BRAF* (at the loci investigated) were found in 11 (100%) of 11 SBT/APST–implant pairs. Overall concordance of *KRAS* and *BRAF* mutations was 95% in 59 of 62 SBT/APST–implant (non-invasive and invasive) pairs ($p < 0.00001$). This study provides cogent evidence that the vast majority of peritoneal implants, non-invasive and invasive, harbour the identical *KRAS* or *BRAF* mutations that are present in the associated SBT/APST, supporting the view that peritoneal implants are derived from the primary ovarian tumour.

Keywords

serous borderline tumour; ovarian low-grade serous carcinoma; ovarian neoplasms

Introduction

There is debate as to whether peritoneal implants associated with serous borderline tumours/atypical proliferative serous tumours (SBT/APSTs) are derived from the primary ovarian tumour or independently (*in situ*) in the peritoneum, reflecting a so-called ‘field effect’. In an effort to resolve this issue, investigators have performed molecular analyses to determine the clonal relationship of the implants to the primary ovarian tumours but results have been conflicting, probably because of the small number of cases studied and because of technical limitations in the various methodologies that have been employed [1–7]. We undertook the current study to determine whether the SBT/APST, referred to herein as APST, and associated peritoneal implants were clonally related. In order to accomplish this goal, we performed a sequencing analysis of the mutation hot spots of *KRAS* and *BRAF* on microdissected samples. In addition, we performed VE1 immunostaining on tissue sections from a small number of primary tumours and implants for which there was insufficient tissue for molecular genetic analysis. The VE1 antibody has been shown by others, and confirmed in the present study, to detect the V600E mutant *BRAF* with a high sensitivity and specificity [8–10]. *KRAS* and *BRAF* genes were selected for analysis because these genes have been shown by whole-exome sequencing to be the most frequently mutated genes in APSTs and low-grade serous carcinomas [11,12].

Materials and methods

Identification of women with advanced stage APSTs and collection of tissue specimens

We selected a total of 45 cases of advanced-stage APSTs from the files of the nationwide Danish Pathology Data Bank. The cases were selected from an ongoing population-based study of the entire female population of Denmark. Slides and blocks from the primary tumours and implants were obtained from pathology departments throughout Denmark and were reviewed by a panel of two gynaecological pathologists (RJK and RV). The 45 cases represent a subset of the entire cohort for which tissue was available and sufficient for molecular analysis of both the APST and the corresponding implant(s) at the time of this analysis. The 45 cases included 40 pure APSTs and five that were APSTs with low-grade serous carcinoma. The case distribution among those 45 women is summarized in Figure 1. A total of 122 formalin-fixed, paraffin-embedded (FFPE) blocks were obtained from 45

patients, which included 33 patients with unilateral APSTs and 12 with bilateral APSTs. Among the 45 cases a total of 65 implants were detected, with at least one peritoneal implant in each case covering 55 non-invasive implants and 10 invasive. Implants involved the peritoneum, omentum, uterus, fallopian tube serosa and appendix. Non-invasive implants included epithelial and desmoplastic types. The study was approved by the Danish Data Protection Agency and the Danish Scientific Ethical Committee. Acquisition of tissues specimen was also approved by institutional review board at Johns Hopkins Hospital (Baltimore, MD, USA).

Laser capture microdissection and DNA extraction

Fifty-five of 57 APSTs (33 unilateral APSTs plus 12 bilateral APSTs) and 56 of 58 peritoneal implants (10 invasive implants and 48 non-invasive implants) contained sufficient tumour tissue to extract DNA and perform mutational analysis. PCR amplification failed in two APSTs and two implants. The remaining seven peritoneal implants (all were non-invasive) were too small to extract sufficient DNA for molecular analysis (Figure 1). These seven cases, together with the four cases of failure in DNA amplification, were analysed with the VE1 antibody, which we and others have demonstrated correlates closely with V600E mutation of the *BRAF* gene (Figure 1). Tissue sections were placed on membrane slides (Carl Zeiss MicroImaging, Göttingen, Germany) and counterstained with haematoxylin. Selected tumour tissues were laser-microdissected and captured using the PALM laser capture microdissection microscope (Leica Microsystem, LMD 7000). An estimated 500–4000 cells were microdissected for each ovarian or extra-ovarian tumour lesion. After 48 h of proteinase digestion, DNA was extracted from the microdissected samples using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA).

Mutational analysis

As shown by our whole-exome sequencing analysis [11], APSTs and LGSCs contain rare somatic mutations, except in *KRAS* at exon 2, codons 12–13, and *BRAF* at exon 15, codon 600. PCR amplification was performed using genomic DNA from laser-captured microdissected FFPE tissue with the following primers: for exon 15 of *BRAF*, forward 5'-TGCTTGCTCTGATAGGAAAATGA-3', reverse 5'-CCACAAAATGGATCCAGACAAC-3'; for exons 2–3 of *KRAS*, forward 5'-TAAGGCCTGCTGAAAATGACTG-3', reverse 5'-TGGTCCTGCACCAGTAATATGC-3'. Amplified PCR products were sequenced at Beckman Coulter (Danvers, MA, USA) and analysed with the Mutation Surveyor DNA Variant Analysis Software. For all the samples, the sequences were reproduced at least two or three times in repeated analysis. Mutational analysis was successful in 111 (55 APSTs plus 56 implants) of 115 specimens (57 APSTs plus 58 implants). Two APSTs and two implants could not be analysed for their mutation status (Figure 1).

Immunohistochemistry

It has been reported that a monoclonal *BRAF* V600E mutation-specific VE1 antibody is able to differentiate wild-type *BRAF* protein from V600E mutated *BRAF* protein by immunohistochemistry on FFPE tissues of different types of tumours, including epithelial

ovarian cancer and melanoma [9,10]. To test the sensitivity and specificity of VE1 immunostaining in recognizing V600E *BRAF* protein on tissue sections, we selected 13 APSTs and 14 implants with known mutation status of *KRAS* and *BRAF* and performed immunohistochemistry with the VE1 antibody using a previously described protocol [8]. The antibody was purchased from Spring Bioscience (Pleasanton, CA, USA; BRAF V600E, Mouse Monoclonal Antibody, clone VE1). Briefly, after pretreatment with cell conditioner 1, pH 8, for 60 min, sections were incubated with VE1 antibody (1:250 dilution) at 37°C for 32 min. Antibody incubation was followed by standard signal amplification with the Ventana amplifier kit, ultra-wash on a Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AR, USA), followed by counterstaining with haematoxylin. For chromogenic detection, an ultraView Universal DAB detection kit (Ventana Medical Systems) was used. The slides were evaluated and scored as positive when > 10% of the tumour cells showed cytoplasmic staining for VE1. Other percentage cut-offs (> 10%, > 25%, > 50%) were also used.

Statistical analysis

The assessment of sensitivity and specificity of VE1 immunostaining in detecting V600E mutations was performed by counting the number of *BRAF* V600E-mutated tumours that were positive for VE1 immunostaining (sensitivity) and the number of *BRAF* wild-type tumours that were negative for VE1 immunostaining (specificity). The significance in co-occurrence of the same mutational status of *KRAS* and *BRAF* between the primary tumour and concurrent implants was assessed by a permutation test. The test statistics was the number of primary tumour–concurrent implant pairs (out of 62) that agreed to mutational status. To generate a null distribution simulating independence while at the same time preserving the overall distribution of the mutations, the mutational status of the implants was randomly reordered. All statistical tests were two-sided.

Results

Mutational analysis of APSTs

A total of 45 patients were studied which included 40 cases that were pure APST and five that contained foci of low-grade serous carcinoma. Seven patients were associated with invasive implants. We observed that three of five APSTs containing a low-grade serous carcinoma component were associated with invasive implants, while only four of 40 pure APSTs were associated with invasive implants; therefore, APST containing the low-grade serous carcinoma component appears more frequently associated with invasive implants than pure APST ($p = 0.03$, χ^2 test) although a larger sample size would be needed to confirm this result. Figure 2 summarizes the mutation analyses of all APSTs and synchronous peritoneal implants. PCR amplification and mutational analysis were successful in 55 (96.5%) of 57 APSTs/STs (Figure 1). Among those 55 APSTs, *KRAS* mutations were identified in 34 (61.8%) and *BRAF* mutations in 8 (14.5%), while the remaining 13 APSTs (23.6%) were wild-type at the mutation hot spots for both genes. All *KRAS* mutations were found at codon 12, with transition of glycine (GGT) to aspartic acid (GAT) in 20 cases (58.8%), to valine (GTT) in 12 cases (35.3%) and to cysteine (TGT) in the remaining two cases (5.9%). All *BRAF* mutations were represented by V600E mutations. *KRAS* and *BRAF*

mutations were mutually exclusive in all cases (see supplementary material, Tables S1, S3). For one contralateral APST (#28) in which PCR amplification failed, it was possible to detect the presence of *BRAF* V600E mutation with VE1 immunostaining. Taken together, 43 (76.8%) of 56 APSTs had either *KRAS* or *BRAF* mutations. All 12 patients with bilateral tumours had the same *KRAS* and *BRAF* mutation in both tumours (Figure 2). Specifically, eight bilateral tumours contained *KRAS* mutation (patients #4, #5, #6, #7, #9, #10, #18, #23), three bilateral tumours had *BRAF* mutations (patients #28 [VE1 immunostaining], #30, #32) and the remaining one harboured wild-type nucleotide sequences for both genes (patient #38).

Mutational analysis of peritoneal implants

Fifty six (96.5%) of 58 peritoneal implants (with successful laser capture microdissection) could be successfully PCR amplified and analysed for *BRAF* and *KRAS* mutations (Figure 1). Mutations at the hot spots of *KRAS* and *BRAF* were detected in 34 (60.7%) and seven (12.5%) of them, respectively (Figure 2). The remaining 15 (26.7%) implants were wild-type for both genes. The mutations involving codon 12 *KRAS* were substitutions of glycine to aspartic acid (GAT), to valine (GTT) and to cysteine (TGT) in 61.7%, 32.3% and 5.8% of implants, respectively. There was no significant difference in mutation frequency between invasive and non-invasive implants. In seven other implants, mutational analysis could not be performed because of small size of the sample.

Immunostaining of VE1 antibody and correlation with morphological features of VE1-positive APST

Although the VE1 antibody has been consistently shown to be a reliable reagent for detecting V600E *BRAF* mutation in tissue sections in several types of cancer [8–10], we wished to confirm this in the present study by performing immunohistochemistry with the VE1 antibody on 13 APSTs (samples 1–13) and 14 implants (samples 16–29) for which the mutational status of *BRAF* was known. As summarized in Table S2 (see supplementary material), all the specimens (APSTs and implants) with *BRAF* V600E mutations were scored positive for VE1 immunoreactivity, while none of the wild-type *BRAF* specimens was positive for VE1. VE1 expression was homogeneous in most cases except for two cases that showed heterogeneous staining. Overall, we demonstrated a concordance between *BRAF* mutational status and VE1 immunostaining with a specificity and sensitivity of 100% (see supplementary material, Table S2). This finding was independent of the cut-off percentages (positive cells > 10%, > 25%, > 50%) being used. The high concordance of VE1 staining pattern with the mutational analysis allowed us to assess the *BRAF* mutation status using immunohistochemistry in two APSTs (see supplementary material, Table S1, samples 14 and 15) in which PCR amplification had failed and in nine small implants (Figure 1 and Table 1, samples 30–38) in which DNA was insufficient for mutational analysis or PCR amplification had failed. One of the two APSTs (see supplementary material, Table S1, sample 14) was positive for VE1 immunostaining; seven of nine implants (see supplementary material, Table S1, samples 30–36) were also positive. Combining the data from Sanger sequencing and VE1 immunostaining, *KRAS* mutations were identified in 34 (53.9%) and *BRAF* mutations in 14 (22.2%) implants; the remaining 15 (23.8%) implants were wild-type for both genes (Figures 2, 3). Mutations of *KRAS* and *BRAF* were never

detected in the same tumour. Accordingly, 76.1% of implants harboured either *KRAS* or *BRAF* mutations.

Correlation of *KRAS* and *BRAF* mutation status between APSTs and associated implants

Since *KRAS* and *BRAF* mutations are mutually exclusive [11,13], we included VE1-positive implants in the *BRAF* mutant group, with a presumption that *KRAS* was wild-type in those cases, while we excluded VE1-negative implants from the analysis because *KRAS* could be either mutated or wild-type. Among 45 patients studied in this report, there were a total of 62 pairs of APSTs and implants (multiple implants in some cases) (Figure 2; see also supplementary material, Table S3). Comparison of the mutations of the paired APSTs and implant(s) showed the same *KRAS* mutation in 34 (92%) of 37 and the same *BRAF* in 14 (100%) of 14. Wild-type sequences of *KRAS* and *BRAF* were recorded in 11 (100%) of 11 pairs of APST and implant. In addition, there were two more cases that showed with wild-type sequences of *KRAS* and *BRAF* in the APST where mutational analysis was not applicable to the implant, but VE1 immunostaining was negative (#34, #39), and one case with wild-type sequence in the implant and mutational status not assessable in the APST, but VE1 negativity (#45). Overall, the mutational status of 62 pairs of APST and implant was the same in 59 (95.1%) (Figure 2; see also supplementary material, Table S4). In the three discordant pairs of tumour and implant, the APSTs had a *KRAS* mutation but the implants showed wild-type *KRAS* and *BRAF* (see supplementary material, Table S3, #5, #10 and #16). There was 100% concordance for invasive implants, as all 10 invasive implants had the same mutation pattern as the corresponding APSTs. Similarly, almost all (94.2%) of non-invasive implants had the same mutation pattern as the corresponding APSTs. To assess the likelihood that this level of concordance could occur by chance while taking the precise rates of each mutation into account, we applied a permutation test, randomly reordering the mutational status of APST–implant pairs to simulate independence. The maximum level of concordance seen in 100 000 random permutations of the data was 50%, corresponding to $p < 0.00001$.

Similarly, same mutation patterns of *KRAS* and *BRAF* were observed in both ovarian APSTs in all 12 women with bilateral tumours. There were 14 patients with multiple peritoneal implants (two to four implants/patient) (Figure 2). Among them, 12 patients (see supplementary material, Table S3, patients #1, #3, #6, #7, #9, #15, #20, #27, #28, #30, #31 and #35) contained the identical mutations in the APST and all the multiple implants. For the remaining two patients with multiple implants (see supplementary material, Table S3, patients #10 and #16), the primary APST and one of the implants were *KRAS* -mutated, while the second implant was *KRAS* wild-type.

Discussion

Several studies have attempted to elucidate the clonal relationship of APSTs and associated implants using a variety of molecular genetic techniques, including mutational analysis, loss of heterozygosity, allelic imbalance counting and X-chromosome inactivation [1–7] (see supplementary material, Table S5). The results, however, have been conflicting, probably due to technical limitations of the assays and the small number of implants studied (with

most studies including fewer than 10 cases). As compared to previous reports, the current study has several strengths. It is the largest series and there was a centralized pathology review by a panel of gynaecological pathologists, which minimized misclassification. In addition to confirming previous reports demonstrating frequent *KRAS* and *BRAF* mutations in the majority of APSTs, our findings provide compelling evidence that there is a highly significant level of concordance of mutational status of *KRAS* and *BRAF* between bilateral tumours, and between the primary ovarian tumour and the associated implants, regardless of whether they are non-invasive or invasive. Accordingly, our findings strongly suggest that the entire process (bilateral tumours and primary tumour and invasive and non-invasive implants) is clonal, as our statistical analysis shows that it is highly unlikely that co-occurrence of the same mutations is a random event.

A clonal relationship between the primary tumour and invasive implants, evidenced by the finding that all 10 invasive implants had the same mutation as the ovarian tumour, is not surprising, as it has been proposed that invasive implants represent metastatic low-grade serous carcinoma. Non-invasive implants, unlike invasive implants, are generally not considered to be malignant, but may have arisen from cells that were shed from the primary tumour and implanted on peritoneal surfaces, analogous to endometriosis. Since non-invasive implants are very frequently associated with endosalpingiosis, it is also conceivable that they arose not from the ovarian tumour but from endosalpingiosis. Interestingly, cases of endosalpingiosis in lymph nodes in women with APSTs have been reported to contain the same *KRAS* mutation in the ovarian tumour and the endosalpingiosis, lending credence to this idea [14]. To prove this point, it will be necessary to compare the mutational status of the implants with the associated endosalpingiosis.

It has been proposed that peritoneal implants may arise through a 'field effect', as has been described in several types of human cancers including head and neck [15] and transitional cell carcinomas of the urinary tract [16]. In these situations, the multiple tumours, although they develop in discrete locations, arise from a common tissue anlage, for example, squamous epithelium for the head and neck tumours and transitional epithelium for the urinary tract tumours. In contrast, the likely source of the ovarian APST is fallopian tube epithelium [17], which is completely different from the mesothelium lining the peritoneal cavity in terms of morphological, immunohistochemical and molecular features. Accordingly, it is unlikely that a field effect is operative in the case of ovarian tumours and peritoneal implants. Finally, the finding of identical mutations in the great majority of pairs of APST and implant provides persuasive evidence that the ovarian tumour and associated implants are clonally related. Although the above represents our favoured view, another possibility also exists. Because APST is characterized only by a small number of mutation types (mainly *KRAS* and *BRAF*), it therefore remains possible that a common mutagenic process acts on different cell populations within peritoneal tissues to produce the same mutation in more than one site. For example, one could envision that ovarian cortical inclusion cysts and endosalpingiosis in the peritoneum are both derived from fallopian tube epithelium, and that when exposed to a common mutagenic insult, both may acquire the same mutation. This is not considered as clonality in the classical sense, but neither is it a random event.

It is of interest that three cases in which the mutational status of the primary tumour and the implants was discordant displayed wild-type sequences for both *KRAS* and *BRAF* in the implants, while the corresponding APSTs harboured *KRAS* mutations in at least one ovary. No discordance was observed in pairs of tumour and implant when the ovarian tumours were wild-type for either gene. It is conceivable that the wild-type *KRAS* status in these three implants was a false negative. If there was very minimal DNA from the epithelial cells in these implants, the PCR reaction may have amplified normal DNA from stromal cells and inflammatory cells instead of the epithelial cells, which would explain the presence of wild-type *KRAS* and *BRAF*. This scenario might also explain the low concordance of mutational status between APSTs (SBTs) and peritoneal implants in previous studies (see supplementary material, Table S5).

In the current study, we found that six (13%) of 45 patients had *BRAF* mutations in APSTs with peritoneal implants. This frequency is considerably lower than the 31.5% in 80 APSTs without peritoneal implants that was previously reported [11,13]. One explanation is that different ethnic backgrounds may account for this difference as the current study recruited only Danish women. On the other hand, it might reflect the limitations of the sample size in our study. Another possibility is that all APSTs in the current report are of advanced stage (ie with peritoneal implants), whereas the APSTs reported in our previous studies were mostly of early stage. If this result can be confirmed, it suggests that *BRAF* mutations may have an inhibitory effect on disease progression. Although the number of *BRAF* mutated cases is small, we did not detect *BRAF* mutations in any of the invasive implants in the current analysis. The underlying biology behind this phenomenon is unclear. Perhaps *BRAF*-mutated APSTs do not disseminate to the same extent as do APSTs with wild-type *BRAF*.

Finally, we found that immunohistochemistry using the VE1 antibody has high sensitivity and specificity for the detection of V600E *BRAF* mutations in FFPE tissue sections. Therefore, this method may be used as a surrogate for mutational analysis in small lesions in which there are insufficient cells of interest to perform molecular genetic analysis.

In summary, this study provides cogent evidence that the vast majority of peritoneal implants, non-invasive and invasive, harbour the identical *KRAS* or *BRAF* mutations that are present in the associated APST. Although we are unable to definitively state that peritoneal implants associated with APSTs are derived from the ovarian tumour, since a mutation at any of the hot spots of *KRAS* and *BRAF* may be coincidental, our statistical analysis indicates that this is highly unlikely to be a random event. We conclude that non-invasive and invasive implants associated with APSTs are very likely derived from the ovarian tumour.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Breakdown of cases in which mutational analysis was performed and those requiring VE1 immunostaining because of failure of PCR amplification or failure of laser capture. PCR-f, PCR failure; LCM-f, laser-capture microdissection failure due to scant lesional cells on tissue sections; VE1 IHC, VE1 immunostaining

Figure 2.

Summary of overall mutational status in all specimens from 45 women analysed in this study. Unilateral APSTs were present in 33 patients and bilateral APSTs in 12 patients, resulting in a total of 57 APSTs. There were 65 implants (55 non-invasive and 10 invasive), resulting in 65 pairs of APST and implants

Figure 3.

The histological features of three representative non-invasive implants with positive or negative VE1 immunoreactivity. The implants from the upper and middle panels were positive for VE1 staining, whereas the implant from the bottom panel was negative