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Original Paper

In ovarian neoplasms, *BRAF*, but not *KRAS*, mutations are restricted to low-grade serous tumours

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Abstract

Genes of the *RAF* family, which mediate cellular responses to growth signals, encode kinases that are regulated by *RAS* and participate in the RAS/RAF/MEK/ERK/MAP-kinase pathway. Activating mutations in *BRAF* have recently been identified in melanomas, colorectal cancers, and thyroid and ovarian tumours. In the present study, an extensive characterization of *BRAF* and *KRAS* mutations has been performed in 264 epithelial and non-epithelial ovarian neoplasms. The epithelial tumours ranged from adenomas and borderline neoplasms to invasive carcinomas including serous, mucinous, clear cell, and endometrioid lesions. It is shown that *BRAF* mutations in ovarian tumours occur exclusively in low-grade serous neoplasms (33 of 91, 36%); these included serous borderline tumours (typical and micropapillary variants), an invasive micropapillary carcinoma and a psammocarcinoma. *KRAS* mutations were identified in 26 of 91 (29.5%) low-grade serous tumours, 7 of 49 (12%) high-grade serous carcinomas, 2 of 6 mucinous adenomas, 22 of 28 mucinous borderline tumours, and 10 of 18 mucinous carcinomas. Of note, two serous borderline tumours were found to harbour both *BRAF* and *KRAS* mutations. The finding that at least 60% of serous borderline tumours harbour mutations in two members of the ERK-MAP-kinase pathway (*BRAF* 36%, *KRAS* 30%) compared with 12% of high-grade serous carcinomas (*BRAF* 0%, *KRAS* 12%) indicates that the majority of serous borderline tumours do not progress to serous carcinomas. Furthermore, no *BRAF* mutations were detected in the other 173 ovarian tumours in this study.

Keywords: **RAS; RAF; ovary; borderline; tumour; mutation**

Introduction

Neoplasms occur as a result of the accumulation of mutations in genes that are critically involved in cell proliferation, differentiation, and death. Recently, oncogenic forms of *BRAF*, a serine-threonine kinase in the RAS-RAF-mitogen/extracellular signal-regulated kinase (MEK), extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase (MAPK) pathway, have been identified in a variety of tumours including malignant melanomas, and large bowel, lung, thyroid, and ovarian neoplasms [1–8]. All *BRAF* mutations have been identified in two regions of the kinase domain. Eighty-nine per cent of the mutations occurred within or immediately adjacent to the activation segment in exon 15, which protects the substrate binding site; 92% of these were accounted for by the V599E mutation which resulted in replacement of valine by glutamic acid. Mutations were identified less commonly in the G loop, in exon 11, which mediates the binding of ATP [1].

Ovarian serous neoplasms exhibit a range of morphological appearances, namely adenomas, conventional high-grade carcinomas, and intermediate forms termed serous borderline tumours (SBTs) [9]. Current evidence, based on clinical [10] and genetic data [11] (for review see ref 12), argues for the most part against SBTs being precursors of high-grade serous carcinomas (SCAs). However, there is evidence that the genetic background of SBT and SCA is similar. Specifically, Tibiletti *et al* [13] found that abnormalities on chromosome 6 (6q27) are present in benign cysts, SBTs, and high-grade SCAs, suggesting that this aberration is important in the progression

of some SBTs to high-grade neoplasms. In contrast, Davies *et al* [1] reported that *BRAF* mutations in the ovary are largely associated with SBTs, and not with high-grade SCAs.

The aim of this study was to investigate in greater detail the prevalence of *BRAF* mutations in ovarian tumours; to explore the relationship between *BRAF* and *KRAS* mutations in a large series of borderline and invasive ovarian neoplasms; and to unravel further the relationship between these neoplastic subtypes.

Materials and methods

Tissue samples and DNA extraction

Ovarian tumour pathology samples were retrieved from the archive of St Mary's Hospital, University College London Hospital, Leiden University Medical Centre, and Chicago University Hospital. The project was approved by medical ethics committees. Representative paraffin wax-embedded blocks were chosen, following review of the cases by four pathologists (NS, BW, TK, and AMF). Tumour tissue from haematoxylin-stained 10–20 μm paraffin wax sections was assiduously microdissected with a 10 G needle under transmitted light microscopy by surgical pathologists (AMF and NS). Contamination by non-lesional cells was estimated to be no more than 25%. DNA was extracted using standard proteinase K digestion followed by heat inactivation of the enzyme. The DNA concentration was quantitated using Picogreen double-stranded DNA (dsDNA) quantitation reagent (Molecular Probes Europe BV, Leiden, The Netherlands), according to the manufacturer's instructions, and the concentrations ranged between 0.5 and 27 ng/μl.

Mutation screening

BRAF

All samples were screened for the common *BRAF* mutation V599E in exon 15 using SNaPshot analysis according to the manufacturer's instructions (ABI PRISM SNaPSHOT™ Multiplex Kit) (Applied Biosystems, Foster City, CA, USA). This mini-sequencing technique relies on a primer that terminates 5′ of the nucleotide, using ddNTPs, in which a known mutation is being sought. A 103 bp PCR product was amplified (annealing temperature 53 °C, 1.3 mM $MgCl_2$) using the following primers: 5′-GAA GAC CTC ACA GTA AAA ATA G (sense primer) and 5′-TCC ACA AAA TGG ATC CAG AC-3′ (antisense) [14]. This product was used as a template for the SNaPshot reaction, the primers for which were 5′-TGA TTT TGG TCT AGC TAC AG-3′ (sense) and 5′-AAA AAA AAA AAC CCA CTC CAT CGA GAT TTC-3′ (antisense).

Direct sequencing for mutations in *BRAF* between nucleotides 1750 and 1810 (exon 15) was performed using a 130 bp PCR product that was amplified using primers 5′-CCA CAG AGA CCT CAA GAG TA-3′ (sense) and 5′-GAA TCC AGA CAA CTG TTC AA-3′. The sequencing PCR reaction was performed using the same primers as those employed in the initial PCR reaction and direct sequencing was performed using a dRhodamine dye terminator sequencing kit (Applied Biosystems) in forward and reverse directions in a capillary semi-automated sequencer (ABI PRISM 377 DNA sequencer) (Applied Biosystems).

Positive controls for these analyses included the A673 Ewing sarcoma cell line and RPMI-7951, a melanoma cell line that contains the V599E *BRAF* mutation. Negative controls included lymphocyte DNA from healthy individuals and commercially produced normal thyroid DNA.

KRAS

All cases were screened for mutations in *KRAS*, exon 1 by direct sequencing. A 116 bp PCR product was amplified using sense and antisense primers: 5-GGC CTG CTG AAA ATG ACT GA-3′ and 5′-GTT GGA TCA TAT TCG TCC AC-3″ (annealing temperature 53 °C, 1.6 mM $MgCl_2$). Low-grade serous neoplasms (SBT, typical and micropapillary variants, and psammocarcinoma) and mucinous neoplasms were also sequenced using a previously reported more sensitive nested PCR-based protocol [14]. In brief, a flanking 179 bp PCR product was amplified (annealing temperature 58 °C) using the primers 5′-AGG CCT GCT GAA AAT GAC TGA ATA-3′ (sense primer) and 5′-CTG TAT CAA AGA ATG GTC CTG CAC-3′ (antisense primer). The resulting fragment was used as a template to amplify a 114 bp fragment including codons 12 and 13 using the primers 5′-AAA ATG ACT GAA TAT AAA CTT GTG G-3′ (sense primer) and 5′-CTC TAT TGT TGG ATC ATA TTC GTC-3′ (antisense primer). This PCR was performed using one standard and one biotinylated primer (annealing temperature 50 °C). The PCR product was captured on a streptavidin-coated sequencing comb (Autoload Solid Phase Sequencing Kit, Amersham Pharmacia Biotech, NJ, USA) and the non-biotinylated strand was removed by alkaline denaturation. The remaining immobilized strand served as the template for dideoxy sequencing reactions using a Cy5 labelled primer (5′-CTC TAT TGT TGG ATC ATA TTC GTC CAC-3′) and T7 DNA polymerase (according to the manufacturer's instructions) and was analysed on the ALFexpress II DNA Analysis System using ALF win Administration software (Amersham Pharmacia Biotech).

All sequencing data were analysed blind with respect to the histopathological diagnoses.

Results

Three hundred and eighty-nine samples from 264 ovarian neoplasms were screened in the first instance for the presence of the V599E *BRAF* mutation using SNaPshot™. This mini-sequencing technique revealed

Table 1. All of the *BRAF* mutations were the common V599E variant in exon 15. No other *BRAF* mutations were identified in either exon 15 or exon 11 in the 264 neoplasms analysed. The *KRAS* mutations were in codon 12 in all but two cases. One was a SBT, typical variant, with a C → T mutation in codon 12 in addition to a G → C mutation in codon 13; the other was a high-grade serous carcinoma with a G → A mutation in codon 13

Tumour type (No. of cases)	*BRAF* mutation Exon 15 (%)	*KRAS* mutation Codon 12 (%)	G → C (%)	G → T (%)	G → A (%)	C → T (%)
SBT, typical variant (82)	29 (35%)	24 (29%)	1 (4%)	9 (38%)	13 (54%)	1 (4%)
SBT, micropapillary variant (6)	3 (50%)	2 (33%)	—	1 (50%)	1 (50%)	—
SBT with an invasive implant (1)	1 (100%)	0	—	—	—	—
Invasive micropapillary carcinoma (1)	0	0	—	—	—	—
Psammocarcinoma (1)	1 (100%)	0	—	—	—	—
Serous carcinoma, high-grade (49)	0	6 (12%)	1 (17%)	3 (50%)	1 (17%)	1 (17%)
Mucinous adenoma (6)	0	2 (33%)	1 (50%)	1 (50%)	—	—
MBT (28)	0	22 (79%)	2 (9%)	10 (45%)	10 (45%)	—
Mucinous carcinoma (18)	0	10 (56%)	3 (30%)	4 (40%)	3 (30%)	—
Endometrioid carcinoma (20)	0	0	—	—	—	—
Clear cell carcinoma (14)	0	0	—	—	—	—
Granulosa cell tumour (10)	0	1 (10%)	—	—	1 (100%)	—
Fibroma/thecoma (20)	0	0	—	—	—	—
Other (8)	0	0	—	—	—	—

MBT = mucinous borderline tumour.

that the V599E mutation was only identified in serous neoplasms and was restricted to tumours of low malignant potential (Table 1). Subsequently, direct sequencing of a 103 bp product of exon 15 from the SBTs (typical and micropapillary variants with and without implants, psammocarcinoma, and invasive micropapillary serous carcinoma) failed to reveal further mutations in these tumours. The regions in exon 15 analysed by sequencing included all the previously reported mutations, other than A1739G. We show for this first time that the common *BRAF* mutation is not present in ovarian stromal neoplasms (Table 1).

Three hundred and sixty-four ovarian samples were analysed by direct sequencing in the first instance for mutations in *KRAS*, exon 1; one sample per case was analysed for all cases, except for the serous and mucinous neoplasms, in which all samples were analysed. All but two mutations were detected in codon 12. One typical variant of SBT contained a mutation in *KRAS* codon 13 (G → C); this tumour also harboured a codon 12 mutation (C → T) (Table 1). The second case was a high-grade serous carcinoma in which a *KRAS* mutation in codon 13 (G → A) was detected. No additional *KRAS* mutations were identified in the low-grade serous or mucinous neoplasms using the more sensitive nested PCR-based sequencing technique [14].

One typical variant of SBT and one micropapillary variant of SBT with lymphatic invasion but without invasive implants harboured both a V599E *BRAF* and a *KRAS* mutation. One tissue block was analysed from the former, a 10 cm tumour with minimal morphological borderline features. The *KRAS* mutation was a G → T mutation in codon 12. This lesion was confined to the inner aspect of one ovary. A *KRAS* G → A mutation in codon 12 was present in the micropapillary variant of SBT, from which only one tissue block was analysed. There were no unusual histopathological features in these cases that distinguished them from the remaining SBTs. Less than 1 year's follow-up is available on these cases but, to date, the patients remain disease-free.

Only one tissue block was analysed for mutations in 52 low-grade serous neoplasms (typical and micropapillary variants, one case with an invasive implant, the psammocarcinoma, and the micropapillary SCA). Between two and nine tissue blocks were examined from the remaining 39 cases; tumour from more than one site was analysed in 14 of these cases. *BRAF* V599E mutations were identified in both ovaries in two cases and four sites in one case, and *KRAS* mutations (codon 12, G → T) were identified in two, three, four, and five sites in a further four cases. Mutational concordance was found in all of these cases. Seven cases in which tumour was analysed from more than one site were uninformative because neither *BRAF* nor *KRAS* mutations were identified. Twenty-eight cases had mutational analysis performed from more than one block from the same ovary (some of these cases also had blocks taken from more than one site) (two blocks,

17 cases; three blocks, eight cases; four blocks, two cases; and six blocks, one case). All samples from any case showed complete mutational concordance with respect to *KRAS* and *BRAF*.

The *BRAF* and *KRAS* mutations in serous neoplasms were not associated with any distinguishing morphological features (Table 1) and did not correlate with stage of disease.

Discussion

This study provides new evidence and confirms previous reports [1,15] that *BRAF* mutations occur in a significant proportion of ovarian low-grade serous neoplasms: specifically, they were identified in 35% (29 of 82) of the typical variant of SBT; in 50% (3 of 6) of the micropapillary variant; and in one invasive implant. A *BRAF* mutation was also identified in another type of low-grade serous neoplasm, namely a psammocarcinoma [16]; analysis of a large group of this sub-type is essential before the significance of this finding can be evaluated. We also found that an equivalent number of SBTs (typical and micropapillary variants) harboured *KRAS* mutations (26 of 91, 29.5%), thereby confirming the previous report [7]. Moreover, no *BRAF* mutations in exon 15, other than the common V599E mutation, were identified in our large series and furthermore, we could demonstrate no mutations in exon 11.

The evidence accrued from our series of SBT, and that of others, indicates that the majority of SBTs do not progress to SCA since approximately 60% of the former have mutations in *BRAF* or *KRAS*, while only 12% of the latter have such mutations. Of note is the finding that no *BRAF* mutations were detected in SCA. This implies that SBTs are, for the most part, unrelated to high-grade SCA and arise by independent molecular routes. Nevertheless, mutations in *KRAS* and *BRAF* were not identified in approximately 30% of SBTs. The possibility therefore still exists that this subset of cases progresses to high-grade SCA; that is, they could both share an as yet unidentified common genetic mutation. In addition, neither *KRAS* nor *BRAF* was identified in other high-grade epithelial neoplasms including clear cell and endometrioid carcinomas. A *KRAS* mutation was identified in a single stromal neoplasm (a granulosa cell tumour).

In general, it appears that *BRAF* mutations occur in the same tumour classes as *RAS* mutations, for example malignant melanoma, colorectal cancer, papillary thyroid cancer, and cholangiocarcinoma. Our results from SBTs indicate that there are no distinguishing morphological features or differences in the clinical staging associated with *BRAF* or *KRAS* mutational status. These findings and the prior knowledge that a direct interaction between *BRAF* and *KRAS* oncoproteins exists in the ERK-MAP-kinase pathway support the hypothesis that *BRAF* and *RAS* mutations employ biologically similar mechanisms to induce neoplasia. The fact that *BRAF* and *RAS* mutations hardly ever occur in the same tumour sample lends further support to this notion [1–8]. Our finding of both *BRAF* V599E and *KRAS* in two tumours was therefore surprising, although there has been one previous report in melanocytic lesions [4]. However, our results need to be interpreted with caution. Although the *BRAF* and *KRAS* mutations in the two SBT samples may have co-existed in the same neoplastic cells, it is not possible to exclude the possibility that they were present in two different neoplastic clones.

Mutational analysis was performed on more than one tissue block from the same ovary in 28 SBTs (see the Results section for details). All the samples from any case showed complete mutational concordance with respect to *KRAS* and *BRAF*. These findings confirm our previous report [17] and the report by Diebold *et al* [18] on the monoclonal origin of multifocal SBT.

BRAF mutations were not detected in any of the 52 mucinous ovarian neoplasms analysed, despite the high incidence of *KRAS* mutations in these lesions, including 33% (2 of 6) of mucinous adenomas, 79% (22 of 28) of mucinous borderline tumours, and 56% (10 of 18) of invasive mucinous adenocarcinomas. This genetic profile, which was also found by others [19], argues in favour of an adenoma–borderline–carcinoma sequence in primary ovarian mucinous neoplasms.

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