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Mutation Analysis of *RASK* and the 'FLR Exon' of *NF1* in Sporadic Ovarian Carcinoma

W.D. Foulkes, P. Englefield and I.G. Campbell

Frequent loss of heterozygosity has been described on several chromosomes in ovarian carcinoma (OC), but few tumour suppressor genes (TSGs) have been analysed. Mutations in the GTPase-related domain (GRD) of the TSG *NF1* have been described in tumours not usually associated with neurofibromatosis type 1 (*NF1*). We analysed 36 OCs for mutations in this domain using single-strand conformation polymorphism. The *NF1*-GRD can downregulate the active form of $p21^{RAS}$ and, therefore, we analysed the same tumours for mutations in *RASK*. No cases of mutations in *NF1*-GRD were seen, and only two cases of *RASK* mutations were found. Thus, activation of the *RAS* signalling pathway by *RASK* or *NF1* mutations does not appear to be common in OC.

Key words: ovarian carcinoma, neurofibromatosis, mutation, tumour suppressor genes
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INTRODUCTION

HUMAN CANCER is characterised by a series of clonal genetic events. Mutations may activate cellular oncogenes, such as *RAS* and *MYC*, or inactivate tumour suppressor genes (TSGs), such as *TP53*, *RB-1* or *APC*. Activation of oncogenes and more especially inactivation of TSGs may be accompanied by loss of heterozygosity (LOH) for the region within which the gene lies. The cascade of events that occur during ovarian carcinogenesis has not been well characterised. Although numerous studies of LOH in ovarian carcinoma (OC) have been reported, of the known TSGs, only *TP53* has been found to be frequently mutated [1–5]. The *RAS* family of oncogenes has been shown to be mutated or amplified in many human cancers [6], but in OC this appears to be uncommon (except perhaps in mucinous carcinomas and borderline tumours) [5–8]. One reason for this discrepancy might be that other components of the RAS signal transduction pathway may be aberrant.

We have shown that in OC, LOH frequently involves all informative markers mapping to chromosome 17 [9]. This implies homo- or hemizygosity is present for TSGs mapping to chromosome 17 such as *TP53* and *NME1*, as well as the putative but as yet uncloned familial breast ovarian cancer gene, *BRCA1*. In addition, *NF1*, the gene which is mutated in neurofibromatosis type 1, is probably a TSG because nearly all the germline mutations seen (translocations, deletions, point mutations and a *de novo* insertion) are consistent with inactivation of the normal gene product [10].

The gene product of *NF1*, neurofibromin, contains a GTPase activating protein ($p120^{GAP}$) related domain (GRD) [11], which is thought to act upstream as a downregulator of $p21^{RAS}$, by promoting its GTPase activity, converting the active, GTP-bound form of $p21^{RAS}$ to its inactive, GDP-bound state [12]. In some tumours, such as colon carcinoma, GTP-bound $p21^{RAS}$ is growth promoting [13]. Mutations in *NF1*-GRD may result in $p21^{RAS}$ persisting in the active form and hence cell growth may result. In some tumours from *NF1* patients, there is no expression of *NF1*-GRD and there are high levels of GTP-bound $p21^{RAS}$ despite normal levels of $p120^{GAP}$ [14]. Others have suggested that *NF1* can act downstream of $p21^{RAS}$, in a manner independent of its GAP-like activity [15].

Theoretically, inactivating mutations could occur anywhere within *NF1*, but if they affect RAS signalling, they are likely to occur in the *NF1*-GRD. Therefore, 36 OCs were analysed by single-strand conformation polymorphism (SSCP) to search for mutations in the highly conserved 159 base pair FLR exon of *NF1*, which is known to contain somatic missense mutations in some human cancers not normally associated with *NF1* [16]. As *NF1*-GRD mutations would be expected to occur in tumours without activating *RAS* mutations, we also used SSCP to analyse the same group of tumours for mutations in *RASK*.

MATERIALS AND METHODS

Histopathology and DNA extraction

Histopathological categorisation, tumour grading and DNA extraction have been described previously [17]. Briefly, tumours were classified and graded using standardised criteria. Of the 36 OCs, there were 22 serous adenocarcinomas, five mucinous adenocarcinomas, four endometrioid carcinomas, four undifferentiated adenocarcinomas and one mixed Müllerian tumour. Cryostat sections were taken from each tumour to ensure that stomal "contamination" was kept to a minimum. DNA was then extracted from the tumours and lymphocytes using the salt-chloroform technique.

SSCP: polymerase chain reaction (PCR) and acrylamide gel conditions

PCRs using the *RASK* and *NF1* 'FLR exon' primers were carried out as previously described [16,18], with some modifi-

Correspondence to W.D. Foulkes at the Human Immunogenetics Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, U.K.
P. Englefield and I.G. Campbell are at the Department of Obstetrics and Gynaecology, University of Southampton, Princess Anne Hospital, Coxford Road, Southampton, SO9 4HA, U.K.
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cations. Approximately 200 ng of DNA were used in a reaction volume of 25 μl. The final concentrations were 1 μM of primers, 0.12 mM of nucleotides and 0.02 μCi of α-[^{32}P]dCTP. The PCRs were diluted 1:3 in SSCP dilution mix (0.1% SDS, 10 mM EDTA, 10 mM Tris). Three microlitres of the reaction were mixed with 4 μl of SSCP loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were heated to 94°C for 10 min and placed on wet ice before loading on to 6% non-denaturing polyacrylamide gels (59 g acrylamide, 1 g bisacrylamide) containing 5% glycerol and 0.5 × TBE. If mutations were not seen under these conditions, the reaction products were run again (in 1 × TBE) in two further gels, one without glycerol and one with 10% glycerol. Electrophoresis was carried out overnight at between 150 and 350 V. After electrophoresis, the gels were transferred to Whatman 3 mm paper, vacuum dried and exposed to Kodak X-AR film overnight at room temperature.

Direct sequencing

RASK PCR products were sequenced using a modification of standard methods [19]. The sequencing gels were fixed in 10% methanol, 10% acetic acid, vacuum dried and exposed to Kodak X-AR film overnight at room temperature.

RESULTS

The highly conserved 'FLR exon' of *NF1* was screened for mutations using SSCP, and no band shifts were seen in the 36 OCs in this series. As mutations in *RAS* lead to its activation, the same tumours were also screened by SSCP for *RASK* mutations. Two OCs showed band shifts, and by direct sequencing, a *RASK* mutation was seen in both cases. The tumours were both grade 1 mucinous adenocarcinomas. The mutations affected codon 12 (Figure 1). Paired lymphocyte DNA was sequenced in tandem, and in both cases the sequence was wild type. Thus, *RASK* mutations were seen in two of five (40%) of the mucinous adenocarcinomas in this series.

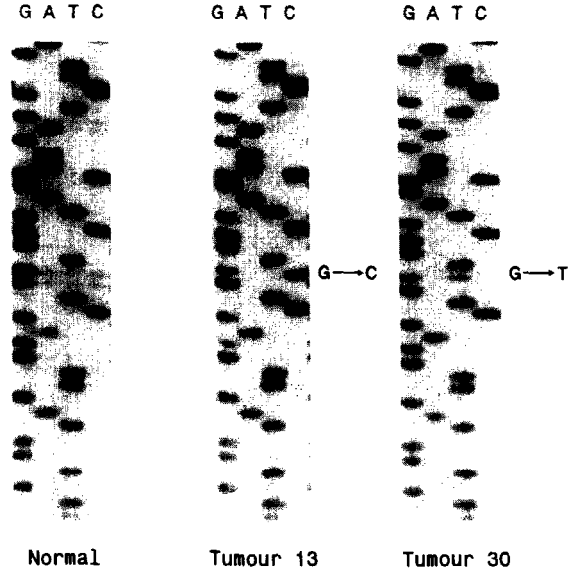

Figure 1. Mutations in *RASK*. 'Normal' (column 1) indicates DNA from the lymphocyte pair of tumour 13. A single base pair substitution in codon 12 was seen (columns 2 and 3); tumour 13: GGT-GCT; tumour 30: GGT-GTT. A faint band from the wild type allele can be seen in both cases.

DISCUSSION

The 'FLR exon' of *NF1* corresponds to codons 1371–1423 of the open reading frame of the *NF1* full-length cDNA [16]. The protein segment encoded by this exon shows ~30% homology to the corresponding regions of human GAP and the yeast homologues IRA1 and IRA2 [11]. The three amino acid FLR motif, near the middle of the exon, is completely conserved in all four proteins and has been shown to be mutated in some human cancers [16]. Therefore, if mutations in *NF1* affecting RAS signalling are present in OC they are likely to be located here. As no mutations were seen when the samples were run on gels containing 0.5 and 10% glycerol (conditions unlikely to miss mutations [20]), NF1- mediated changes in RAS signalling are not likely to be important in OC.

In this study we have shown that *RASK* mutations are far more common in mucinous OCs than in serous OCs (two of five versus zero of 22). This finding is consistent with previous reports [5, 8]. It is interesting to note that mucinous and serous carcinomas appear to have a different genetic profile. For example, we [9] and others have shown that LOH on chromosome 17 is rarely seen or absent in mucinous carcinomas, whereas up to 80% of serous carcinomas show such change. This implies that either specific genetic changes define the morphology of the tumour, or that the developmental path a tumour follows renders it susceptible to different genetic aberrations.

It appears that perturbations in the RAS signalling pathway do not have an important role in OC. Apart from *TP53*, very little is known about TSGs in OC. Mutations do not appear to be present in the Wilms' tumour gene, *WT1* [21] or in the first 4.7 kb of the cDNA encoding APC [22]. There are no published reports of mutations in *RB-1*, *DCC*, *NF2* or *VHL* in OC. The familial breast ovarian cancer gene, *BRCA1*, mapping to 17q12-21 obviously will have an important role in OC, but it is likely that, as in colorectal cancer, many different steps will be needed for carcinogenesis [23]. We have shown that mutations in the 'FLR exon' of *NF1* do not appear to be part of this sequence of events, and that *RASK* mutations are only common in mucinous ovarian carcinomas.

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A Combination of Subcutaneous Recombinant Interleukin-2 and Recombinant Interferon-α in the Treatment of Advanced Renal Cell Carcinoma or Melanoma

Merisisko Vuoristo, Ismo Jantunen, Seppo Pyrhönen, Timo Muhonen and Pirkko Kellokumpu-Lehtinen

In this phase II study, we have evaluated the efficacy and toxicity of low-dose subcutaneous (s.c.) recombinant interleukin-2 (IL-2) and recombinant interferon (IFN)-α in 16 patients with advanced renal cell carcinoma (RCC) and in 4 patients with advanced melanoma. The complete course on this protocol comprised 6 weeks of s.c. IL-2 plus IFN-α followed by a 2-week rest period. The treatment was moderately strenuous for patients, requiring frequent dose reductions; only eight cycles (30%) could be administered to 75–100% of the projected dose. Main side-effects were fever, fatigue, hypotension, liver toxicity, neurotoxicity and skin reactions. Among the evaluable 17 patients, two responses (one partial, one complete) were seen in patients with RCC. This regimen proved to be rather toxic and yielded a modest response rate of 15% in RCC, but initial findings concerning the duration of survival seem promising.
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INTRODUCTION

THE RESULTS of treatment of advanced melanoma and renal cell carcinoma (RCC) have remained far from satisfactory. Chemotherapy alone yields response rates of approximately 20% in melanoma, and 10% in RCC [1, 2]. Interferon (IFN)-α has been used at various doses for RCC and melanoma, with response rates of 15–20% [3–5]. Interleukin 2 (IL-2) entered clinical trials in 1985, when Rosenberg and his coworkers published their observations on treatment with IL-2 combined with lymphokine activated killer (LAK) cells in various cancers [6]. The responses seen were most promising in RCC and melanoma. However, high doses of intravenous IL-2 caused major side-effects in the