



MDM2 overexpression is rare in ovarian carcinoma irrespective of *TP53* mutation status

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Summary Somatic mutations in *TP53* are seen in many human cancers. In addition, the protein product of the wild-type *TP53* can be sequestered by the protein MDM2 (murine double minute 2). This protein is commonly overexpressed in human sarcomas and gliomas, usually as a result of gene amplification. In this study, 43 ovarian carcinomas (OCs) were analysed for aberrations in the *TP53* gene by immunohistochemistry (IHC), loss of heterozygosity (LOH) or mutation analysis. The *MDM2* gene and its product was studied by Southern blotting and IHC. Over 50% of the OCs studied showed mutations in *TP53* by either direct sequencing (19/36, 53%), positive IHC (23/43, 53%) or both, whereas 0/32 had amplification of *MDM2* and only 1/37 tumours had positive IHC using the anti-MDM2 antibody IF-2. The solitary example of positive IHC in this series was seen in a mixed müllerian tumour with sarcomatous differentiation and was not accompanied by *MDM2* DNA amplification. These results support previous data showing that around 50% of OCs have mutations in *TP53* and in addition, suggest that *MDM2* is not amplified in OC, but the presence of sarcomatous features in mixed müllerian tumours may result in positive immunohistochemistry with IF-2.

Keywords: ovarian cancer; MDM2; TP53; mutation; immunohistochemistry

Somatic mutations in *TP53* are very common in human cancer (Hollstein *et al.*, 1991). This 393 amino acid nuclear phosphoprotein, mapping to 17p13.1, is active as a transcription factor and binds DNA in a sequence-specific manner. Baker *et al.* (1989) showed that mutations in the *TP53* gene of two colorectal cancers affected a highly conserved region of the gene, and was associated with allelic losses of the wild-type *TP53* gene. This suggested that *TP53* acts as a tumour-suppressor gene (TSG). These findings were later generalised to a number of human cancers (Nigro *et al.*, 1989), but it was emphasised in this paper that mutation of *TP53* was not always associated with LOH on 17p.

Previous studies of *TP53* in sporadic OC have shown that mutations are present in up to 80% of OCs (Marks *et al.*, 1991; Mazars *et al.*, 1991; Okamoto *et al.*, 1991; Kohler *et al.*, 1993a,b; Kupryjanczyk *et al.*, 1993; Milner *et al.*, 1993; Teneriello *et al.*, 1993). Taken together, these studies demonstrate that

- (1) Mutation of *TP53* is usually accompanied by overexpression of TP53 protein as detected by most of the anti-p53 monoclonal antibodies and vice versa, but there are a number of tumours where this relationship does not hold.
- (2) Mutation of *TP53* is commonly associated with LOH of the wild-type allele, but often there is LOH over the whole of chromosome 17.
- (3) *TP53* mutation occurs before metastatic spread, and is less common in early-stage than late-stage disease.
- (4) There is no strong evidence that any particular type of mutation (transition, transversion, mutation hotspot) is overrepresented in OC.
- (5) Mutations in *TP53* and *RAS* are not seen in the same tumour.
- (6) There is no clear prognostic information to be gathered from the presence of a *TP53* mutation in any given OC.

An amplified murine gene, *mdm2* (murine double minute 2) was cloned from a transformed mouse 3T3 cell line (Cahilly-Snyder *et al.*, 1987). The human homologue, *MDM2*, mapping

to 12q13–14, is amplified in a number of human cancers, particularly sarcomas and gliomas (Oliner *et al.*, 1992; Leach *et al.*, 1993; Reifenberger *et al.*, 1993). The product of this gene complexes with wild-type *TP53* and can abolish its *trans*-activating properties (Momand *et al.*, 1992), probably by masking the activation domains of TP53 (Oliner *et al.*, 1993). Therefore, in a given tumour, one might expect to see mutations of *TP53*, with or without LOH on 17p, or amplification of *MDM2*, but not both. This has been shown to be the case for soft tissue sarcomas and gliomas (Leach *et al.*, 1993; Reifenberger *et al.*, 1993).

As the relationship between *TP53* and *MDM2* has not been analysed in OC, in this study of 43 OCs we set out to search for mutations in *TP53*, as identified by single-strand conformation polymorphism (SSCP) analysis of exons 5–8, direct sequencing and IHC, and amplification and overexpression of *MDM2* in the same OCs.

Materials and methods

Histopathology, DNA extraction and Southern hybridisation

Histopathological categorisation, tumour grading, DNA extraction and Southern hybridisation were carried out as described in our previous publication (Foulkes *et al.*, 1993a). Table I shows the histopathological subtype of each tumour studied. Cryostat sections were taken from each tumour to ensure that stromal 'contamination' was kept to a minimum. DNA was then extracted from the tumours and lymphocytes as described previously.

TP53 LOH studies

These were carried out as described in Foulkes *et al.* (1993b). LOH was studied using a *bg*III restriction fragment length polymorphism (RFLP) within the *TP53* gene (De La Calle *et al.*, 1990) and using the highly informative dinucleotide repeat that lies near *TP53* (Jones and Nakamura, 1992). Some of this work has been previously reported (Foulkes *et al.* 1993b) but here we present an extended analysis.

Table 1 Histopathological details, *TP53* mutation. LOH on 17p and p53 and MDM2 immunohistochemistry

Tumour number	Histopathological type	Grade ^a	Stage	TP53 LOH	TP53 ^b IHC	TP53 mutation ^c codon, base change	Mutation type	Amino acid change	MDM2 IHC
11	SPAC	3	NA	+	+	173, GTG→ATG	T	Val→Met	-
32	SPAC	2-3	III	+	-	175, CGC→CAC	CpG	Arg→His	ND
85	SPAC	2	NA	+	+	176-182, 19 bp deletion		frameshift	-
17	SPAC	3	NA	+	+	179, CAT→CGT	T	His→Arg	-
80	SPAC	3	IIIc	+	+	179, CAT→CGT	T	His→Arg	-
10	SPAC	3	NA	+	-	220, TAT→TGT	T	Tyr→Cys	-
24	AC/UD	3	III	+	+	220, TAT→TGT	T	Tyr→Cys	-
42	EC	2	NA	+	+	220, TAT→TGT	T	Tyr→Cys	ND
64	AC/UD	3	II	+	+	220, TAT→TGT	T	Tyr→Cys	-
83	SPAC	3	IIIc	+	+	220, TAT→TGT	T	Tyr→Cys	-
29	SPAC	2-3	III	+ ^d	-	236, TAC→TGC	T	Tyr→Cys	-
20	SPAC	2	NA	+	+	248, CGG→TGG	CpG	Arg→Trp	-
75	MAC	2	IV	-	+FCP	272, GTG→ATG ^e	T	Val→Met	-
7	AC/UD	3	NA	+	+	273, CGT→TGT	CpG	Arg→Cys	-
61	SPAC	2	III	+	+	273, CGT→TGT	CpG	Arg→Cys	-
67	SPAC	3	III	-	+FCP	273, CGT→TGT ^f	CpG	Arg→Cys	-
88	SPAC	3	III	-	+	281, GAC→GGC	T	Asp→Gly	-
37	SPAC	3	II	+	+	282, CGG→TGG	CpG	Arg→Trp	-
48	SPAC	3	II	+	+	282, CGG→TGG	CpG	Arg→Gly	-
47	AC/UD	2	IV	+	+	Mutation, but not identified			ND
9	AC/UD	3	NA	+	+	ND			-
35	EC	3	III	ND	+	ND			-
40	EC	3	III	+	+	ND			-
63	AC/UD	3	NA	NI	+	ND			-
71	SPAC	2-3	III	ND	+	ND			-
84	MMT	3	III	ND	+	ND			-
13	MAC	1	NA	-	-	-			-
18	SPAC	3	NA	ND	-	-			ND
25	SPAC	3	III	+	-	-			-
27	SPAC	3	III	-	-	-			ND
28	SPAC	2-3	NA	+	-	-			-
30	MAC	1	III	-	-	-			-
31	EC	2	III	- ^d	-	-			-
41	SPAC	3	III	+	-	-			ND
50	MMT	3	III	+	-	-			-
51	MAC	1	III	-	-	-			-
53	SPAC	2	IV	+ ^d	-	-			-
72	SPAC	3	IV	+	-	-			-
73	MMT	2	II	NI	-	-			+
77	SPAC	2-3	IV	+	-	-			-
78	EC	2	Ic	-	-	-			-
81	SPAC	3	IV	NI	-	-			-
91	SPAC	3	III	NI	-	-			-

^aFor details of grading system, see Foulkes *et al.* (1993a). ^bp53 Immunohistochemistry was performed with antibodies CM1, PAb 421 and DO7 (anti-p53). ^cMutation type: CpG indicates a GC→AT transition at CpG dinucleotide; T, transition. ^dIn these cases, the two *TP53* probes used were not informative. However, a probe nearby gave the result shown. These cases gave all the same LOH result (+ or -) over the whole chromosome making it likely that, if informative, the *TP53* result would have been as indicated. ^eVery faint band, probably affecting 5% DNA. Percentage tumour/stroma in these two cases is; tumour 67, 40/60%; tumour 75, 50/50%. See text for further discussion. AC/UD, adenocarcinoma, undifferentiated lineage; SPAC, serous papillary (cyst) adenocarcinoma (including serous carcinoma, papillary carcinoma and serous adenocarcinoma); MAC, mucinous adenocarcinoma; EC, endometrioid carcinoma; MMT, mixed müllerian tumour. NA, not available; ND, not done; NI, not informative; FCP, few cells positive.

TP53 SSCP and sequencing analysis

Polymerase chain reactions (PCRs) using the *TP53* primers were carried out as described in Milner *et al.* (1993) with some modifications. After standard treatment, the samples were placed on wet ice before loading onto 6% non-denaturing polyacrylamide gels (59:1 acrylamide:bis-acrylamide) containing 5% glycerol and 0.5 × TBE. Electrophoresis was carried out overnight at between 150 and 350 V. After electrophoresis, the gels were transferred to Whatman 3MM paper, vacuum dried and exposed to Kodak X-AR film overnight at room temperature. If band shifts were not seen under these conditions, the reaction products were run again (in 1 × TBE) in a gel containing 10% glycerol. Any tumours with SSCP band shifts were sequenced using a modification (Powis *et al.*, 1992) of standard method. The sequencing gels were fixed in 10% methanol, 10% acetic acid, vacuum dried and exposed to Kodak X-AR film overnight at room temperature.

Immunohistochemistry: TP53 and MDM2

This was carried out on 4 µm sections from formalin-fixed paraffin-embedded tissue (antibodies CM1 and DO7), as well

as on 6 µm cryostat sections (antibodies PAb 421 and IF-2). The sections were immunostained with these polyclonal and monoclonal antibodies using a standard avidin-biotin peroxidase technique as described previously (Pignatelli *et al.*, 1992). All primary antibodies were incubated at 25°C for 1 h at appropriate dilutions: neat (HMFG2 and α₆-integrin-positive controls), 1:10 (MDM2), 1:100 to 1:200 (DO7, 421) and 1:1000 (CM1). In some cases, CM1 was incubated at a dilution of 1 in 2000 at 4°C overnight. All the stained sections were reviewed by GWHS. Tumours were assessed as positive when there was unequivocal reproducible nuclear staining of at least 5-10% of the neoplastic cells, assessed as weak, moderate or strong. In practice, in the positive tumours, a greater percentage of positive cells were present. Cytoplasmic staining was ignored as false-positive reactions are known to occur (e.g. with CM1). Positive controls were used in all cases, and immunostaining was repeated if there was any uncertainty.

MDM2 genomic amplification analysis

MDM2 gene amplification was assessed by Southern blotting with a 1.6 kb cDNA probe, pHDM (Chen *et al.*, 1993). The

intensity of the autoradiographic signals obtained were compared with DNA loading by comparison with previous hybridisation signals performed on the same blots. Additionally, all blots were hybridised with a probe for the *RET* proto-oncogene (Mulligan *et al.*, 1993), which is a single-copy gene on chromosome 10q11.2.

Results

Mutation, immunohistochemistry and loss of heterozygosity for TP53

We analysed our series of tumours for aberrations in *TP53* by SSCP followed by direct sequencing, IHC with three anti-p53 antibodies and by LOH using a highly polymorphic dinucleotide repeat marker. Using this combined approach we have shown that 19/36 OCs had mutations in *TP53* by DNA sequence analysis, performed as a result of an aberrantly migrating PCR fragment seen on SSCP (Table I). Eighteen of these mutations were missense mutations and one was a deletion (tumour 47 showed a very clear band shift with SSCP analysis, but despite many attempts, this tumour could not be sequenced and therefore this tumour has been included as 'mutation, but not identified'). The 18 base substitutions seen in this series of 35 OCs were all transitions, with 50% being at GC→AT, (39% at CpG sites and 11% at other sites) and 50% were AT→GC transitions. Five out of these 9 AT→GC transitions were at codon 220, TAT→TGT, changing the amino acid sequence from tyrosine to cysteine. Thus no transversions or complex substitutions were seen. The deletion (tumour 85) would result in a frame-shift and therefore would be likely to produce a truncated protein. This case was positive for p53 IHC.

Twenty-three of 43 OCs (53%) were positive by IHC with the anti-p53 antibodies PAb 421, DO7 or CM1. All three antibodies were used where tissue was available, and no discordant results in terms of positive vs negative immunostaining were obtained using these three antibodies. The correlation between mutation in *TP53* and positive IHC was highly significant (Fisher's exact test, $P < 0.001$) (Table II). However, the common mutation in our series is codon 220, TAT>TGT, Tyr>Cys and the IHC results were not completely concordant for these five tumours. The five tumours were no. 10, a serous papillary adenocarcinoma, (SPAC); no. 24, an undifferentiated adenocarcinoma, (AC/UD); no. 42, an endometrioid carcinoma; no. 64, an AC/UD; and no. 83, a SPAC. Tumour 10 showed clear LOH at *TP53* and a mutation in exon 6, as did the other four cases (Table I). Despite the differing histology, all except tumour 10 were positive for IHC with the anti-p53 antibodies used. It is possible that another mutation has occurred in this tumour outside exons 5–8 which prevents protein from being produced, resulting in negative IHC. Alternatively, the protein may have become destabilised in the period between tumour removal and snap freezing, although from our positive results with this tumour using other antibodies (not shown) this seems unlikely. There were two other tumours that had *TP53*

mutations but negative IHC. These were tumours 29 and 32, both serous papillary adenocarcinomas with mutations in *TP53* at codons 236 and 175 respectively (Tables I and II).

A PCR-based analysis of LOH at the *TP53* locus showed that 26/35 (74%) of the informative tumours that were studied at this, or in four cases an adjacent locus, demonstrated LOH. The correlation between *TP53* mutation and LOH is shown in Table III. It can be seen that while there is a good correlation between mutations in *TP53* and LOH at this locus (Fisher's exact test, $P = 0.059$), LOH at *TP53* without mutations in this gene was not uncommon. This is probably because other, as yet uncloned, TSGs on

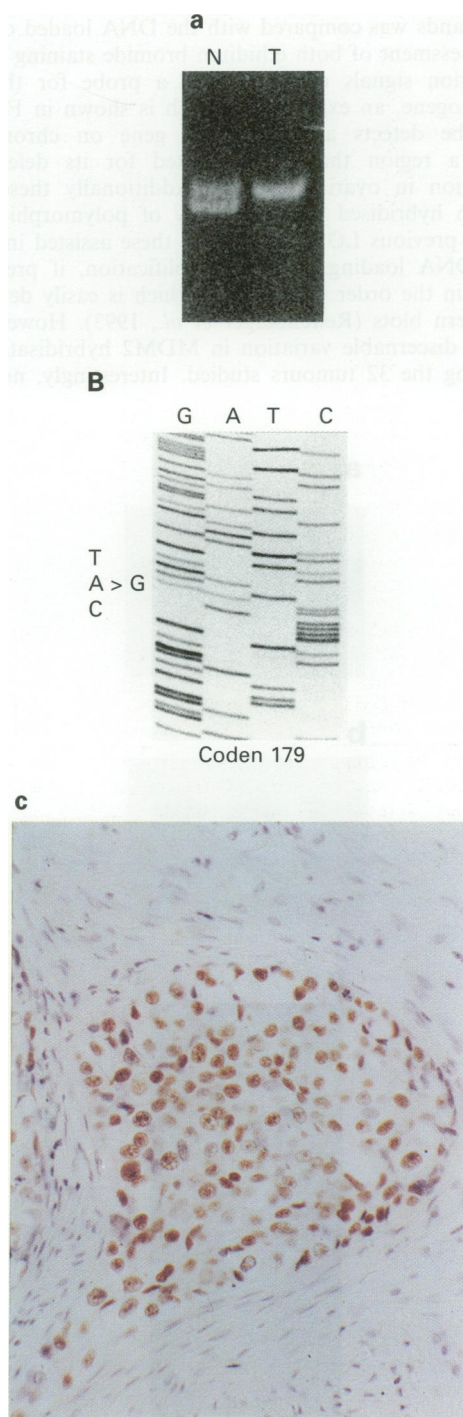


Figure 1 *TP53* loss of heterozygosity, sequencing and immunohistochemistry results for tumour 80. (a) Microsatellite analysis of normal (N) and tumour (T) genomic DNA at the *TP53* locus showing complete loss of the smaller allele in the tumour. (b) DNA sequence of a portion of exon 5 of *TP53* showing an A to G transition at codon 179. (c) Positive p53 immunostaining using antibody DO7.

Table II Comparison between mutations in *TP53* and positive immunohistochemistry

	Positive IHC	Negative IHC
Mutation in <i>TP53</i>	17	3
No mutation in <i>TP53</i>	0	17

Fisher's exact test, $P < 0.001$.

Table III Comparison between mutations in *TP53* and LOH

	LOH at <i>TP53</i>	No LOH at <i>TP53</i>
Mutations in <i>TP53</i>	17	3
No mutations in <i>TP53</i>	7	6

Fisher's exact test, $P = 0.059$.

chromosome 17 are 'driving' the LOH. Similarly, there are three cases with mutations but no LOH. As these are all late stage, moderate-to-high-grade tumours, it seems unlikely that in these cases *TP53* mutation is an early event (with LOH to follow). Examples of LOH at *TP53*, mutation in *TP53* and positive IHC with monoclonal antibody DO7 are shown in Figure 1 (tumour 80). Interestingly, this tumour shows complete LOH but the wild-type genotype is clearly visible on the sequencing gel, suggesting that LOH with reduplication or recombination preceded mutation in *TP53*.

MDM2 amplifications and over-expression

Southern blots of tumour and normal DNA were probed with an *MDM2* cDNA, pHDM. The intensity of the hybridising bands was compared with the DNA loaded onto the gel by assessment of both ethidium bromide staining and the hybridisation signals obtained with a probe for the *RET* proto-oncogene, an example of which is shown in Figure 2. This probe detects a single copy gene on chromosome 10q11.2, a region that is not noted for its deletion or amplification in ovarian cancers. Additionally these filters have been hybridised with a variety of polymorphic DNA probes in previous LOH studies and these assisted in verifying the DNA loading. *MDM2* amplification, if present, is generally in the order of 5–8 fold which is easily detectable on Southern blots (Reifenberger *et al.*, 1993). However, we found no discernable variation in *MDM2* hybridisation signals among the 32 tumours studied. Interestingly, no DNA

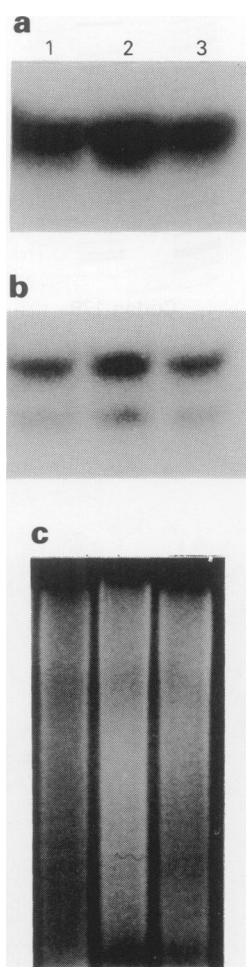


Figure 2 Southern blot (*Bam*HI-digested DNA) analysis of the *MDM2* gene in some representative tumours (a) showing no amplification. There were no significant differences in DNA loading as demonstrated by the signals obtained with a probe for the *RET* proto-oncogene (b) or by comparison with the ethidium bromide stained gel from which the Southern blot was derived (c).

amplification was evident in tumour 73 which was positive for *MDM2* by IHC (see below).

Thirty-seven tumours were examined for *MDM2* protein overexpression by IHC using the antibody IF-2. Only one tumour, 73, showed positive *MDM2* IHC (Figure 3). This tumour was negative for p53 IHC and *TP53* mutation. Interestingly, this was a mixed müllerian tumour with histological features similar to sarcomas, a tumour type where *MDM2* amplification and over-expression is frequently observed.

Discussion

Here we present the first account of a combined approach to the analysis of *TP53* and *MDM2* aberrations in sporadic OC. We have demonstrated that

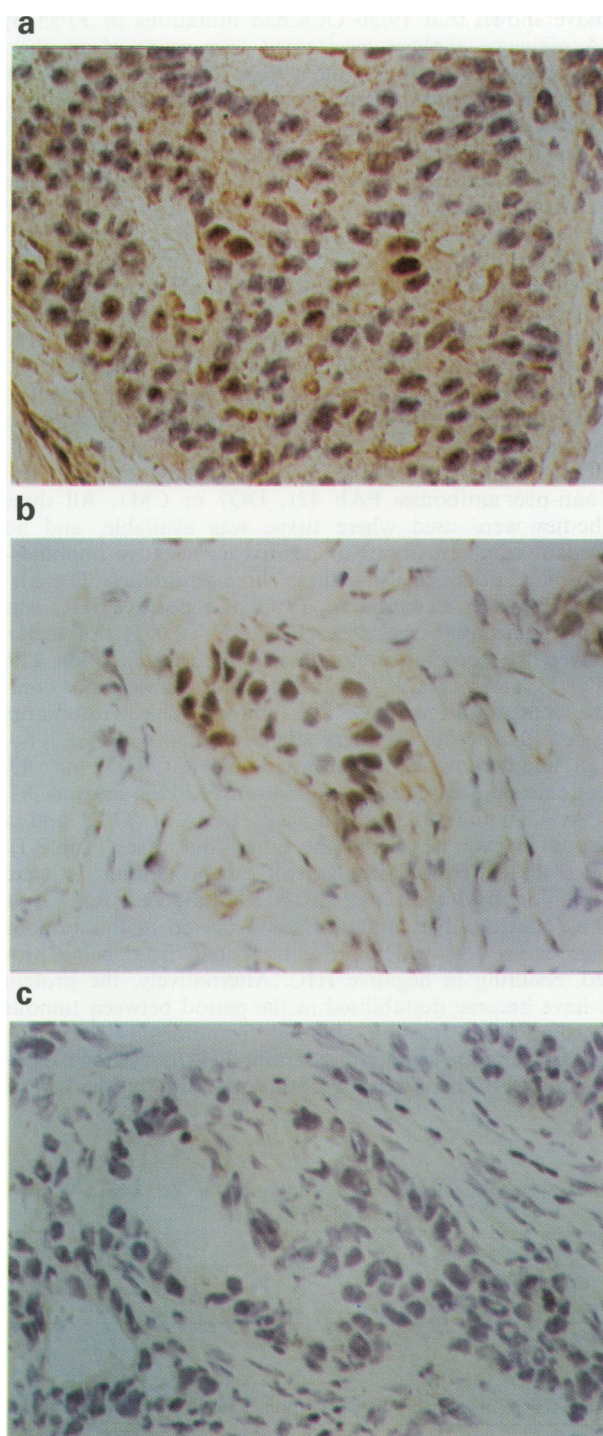


Figure 3 *MDM2* immunohistochemistry using antibody IF-2. (a) Positive *MDM2* immunostaining with tumour 73. (b) Positive *MDM2* immunostaining in a mammary carcinoma. (c) Absence of staining on an endometrioid carcinoma.

- (1) Twenty-six of 35 (74%) informative OCs have LOH on chromosome 17p at the *TP53* locus.
- (2) Nineteen of 36 (53%) OCs have mutations in codons 5–8 of *TP53*.
- (3) Twenty-three of 43 (53%) OCs have positive IHC using anti-p53 antibodies.
- (4) None of 32 OCs had *MDM2* amplification by Southern blotting using the *MDM2* cDNA.
- (5) One of 37 tumours (2.7%) had positive IHC which did not accompany DNA amplification.

The results we have obtained at the *TP53* locus are in keeping with previous studies of OC showing that approximately 50% of all OCs have mutations in *TP53* exons 5–8. The spectrum of mutations we observed differ slightly from the experience of other groups, in that all the missense mutations we found were transitions, whereas in a review of all OC cases reported 93/128 mutations were transitions (73%) and 35/128 (27%) were transversions (Kohler *et al.*, 1993b). The smaller numbers in our series may account for the differences seen. This study supports the conjecture that environmental toxins are unlikely to be responsible for *TP53* mutations in OC (Kohler *et al.*, 1993b).

It has been suggested that mutations in *TP53* and amplification of *MDM2* in the same tumour are biologically redundant, in that both alterations have the same effect: loss of p53 function. Thus, in our series of OCs we studied *MDM2* amplification and over-expression in order to determine whether *MDM2* was aberrant in OC and if so, whether amplification and/or overexpression was limited to those tumours without *TP53* alterations. Of 43 OCs available, 32 were studied by Southern blotting using an *MDM2* cDNA and 37 by IHC using the antibody IF-2. No tumours showed amplification, and only one tumour, a mixed müllerian tumour, demonstrated overexpression. Therefore in OC, regulation of *TP53* does not appear to be mediated by *MDM2* overexpression.

The tumour showing positive IHC with IF-2, tumour 73, is negative for IHC with anti-p53 antibodies and does not have a mutation in exons 5–8 of *TP53*. This mixed müllerian tumour is, to our knowledge, the only ovarian tumour to be described that demonstrates an overexpression of *MDM2* protein. The fact that the tumour displays sarcomatous features is intriguing, as it is soft-tissue sarcoma (Leach *et al.*, 1993; Cordon-Cardo *et al.*, 1994; Florenes *et al.*, 1994a; Patterson *et al.*, 1994), and to a lesser extent gliomas (Reifenberger *et al.*, 1993) and osteosarcomas (Ladanyi *et al.*, 1993; Florenes *et al.*, 1994a), that most commonly show amplification and/or overexpression of *MDM2*. There may be particular features of the differentiation of mesenchymal tissue that favour the accumulation of quantitatively abnormal *MDM2* protein.

The presence of overexpressed *MDM2* in the absence of amplification has been reported in two series of sarcomas (Cordon-Cardo *et al.*, 1994; Florenes *et al.*, 1994a) and, perhaps surprisingly, in 25 out of 87 bladder cancers in one

series (Lianes *et al.*, 1994). In this latter series, only one tumour had DNA amplification and overexpression, which suggests that *MDM2* overexpression in bladder cancer may have different biological implications from that seen in sarcomas, especially as the same group found that overexpression was significantly associated with low-grade, early-stage tumours. The significance of overexpression of *MDM2* without amplification is uncertain: it has been suggested that in this context *MDM2* expression (to levels detectable by IHC) may be a response to wild-type *TP53* (Meltzer, 1994). Our findings with tumour 73 would support this view. However, we did not see any *MDM2* overexpression in the other 16 OCs in our series that lacked mutations in *TP53* exons 5–8.

The spectrum of tumours that show *MDM2* amplification and/or overexpression has been studied by several groups. In general, it appears that *MDM2* amplification and/or overexpression is present in up to one-third of all soft-tissue sarcomas and about 10% of glioma and metastatic osteosarcomas. In contrast, *MDM2* amplification and/or overexpression is very uncommon or absent in colon carcinoma (Oliner *et al.*, 1992), Ewing's sarcoma (Kovar *et al.*, 1993), gastric carcinoma (Oliner *et al.*, 1992), hepatoblastoma (Waber *et al.*, 1993), leukaemia (Ridge *et al.*, 1994), malignant melanoma (Florenes *et al.*, 1994b), myelodysplastic syndromes (Preudhomme *et al.*, 1993), neuroblastoma (Waber *et al.*, 1993), oesophageal carcinoma (Esteve *et al.*, 1993), ovarian carcinoma (this report), upper aerodigestive tract squamous carcinoma (Waber *et al.*, 1993), uterine cervix carcinoma (Kessiss *et al.*, 1993) or Wilms' tumour (Waber *et al.*, 1993). The question of *MDM2* involvement in bladder cancer is unresolved as, though amplification of *MDM2* was rare in two independent studies (2/50, Habuchi *et al.*, 1994; 1/87, Lianes *et al.*, 1994), the latter group found that overexpression without amplification was common (25/87).

Our results confirm the importance of *TP53* in OC. Indeed, aberrant *TP53* expression is one of the commonest genetic defects seen in human cancers (Hollstein *et al.*, 1991). The abnormal expression commonly results from mutations in the coding region of *TP53*. However, the finding that *MDM2*, a protein that binds p53, was amplified in sarcomas that did not show mutations in *TP53* (Oliner *et al.*, 1992) suggested that this might be an alternative way of abrogating p53 function. The importance of *MDM2* in soft-tissue sarcomas is clear, but taking all the published data together it appears that in many human cancers p53 is not commonly inactivated by sequestration through *MDM2* amplification. It may be that *MDM2* has a particular role in mesenchymal tumours, but *MDM2* amplification in the common adult cancers (where *TP53* mutations are frequent) is uncommon.

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