

# p53 alterations are predictive of chemoresistance and aggressiveness in ovarian carcinomas: a molecular and immunohistochemical study

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**Summary** Chemotherapeutic management of ovarian cancers is a difficult task as these neoplasms show significant differences in chemosensitivity, even if they share identical clinicopathological features. The present study was undertaken to investigate the prognostic and predictive role of p53 alterations in ovarian cancer. To this end, using different technical approaches, i.e. genetic and immunohistochemical analyses, we analysed a series of 68 ovarian neoplasms including 15 low malignant potential (LMP) tumours and 53 invasive carcinomas. We never observed p53 abnormalities in LMP tumours. p53 alterations were present only in invasive ovarian carcinomas, and they were detected much more frequently in tumours characterized by high histological grade ( $P=0.01$ ) and advanced-stage disease ( $P=0.006$  and  $P=0.05$  for gene mutations and protein expression respectively). For 33 patients with invasive ovarian cancer, information was available concerning response to cisplatin-based chemotherapy. A strong correlation ( $P=0.001$ ) has emerged between p53 alterations and response to chemotherapy: only one (14%) of seven patients who had a pathological complete response to antineoplastic drugs showed p53 aberrations, whereas 18 (82%) of 22 cases with partial response and all of the four non-responsive patients scored positive for p53 abnormalities. We also observed that patients with p53 mutations had a significantly shorter progression-free survival than patients with p53-negative tumours ( $P=0.05$ ). Taken together, our results strongly suggest that in epithelial ovarian malignancies tumours showing p53 aberrations are significantly less sensitive to chemotherapy and more aggressive than those with functional p53. Thus, a routine analysis of this gene could have profound implications for the treatment of ovarian cancer.

**Keywords:** p53 mutations; p53 expression; polymerase chain reaction; single-strand conformation polymorphism; immunohistochemistry; ovarian carcinoma; *cis*-diamminedichloroplatinum II (cisplatin); chemoresistance

In most malignancies, drug resistance represents a major impediment to the control of neoplastic growth. Ovarian cancer presents an example of difficult chemotherapeutic management. Despite the improvements in therapeutic response, obtained mainly with *cis*-diamminedichloroplatinum II (CDDP)-based chemotherapy, approximately 50% of patients in advanced stage of disease are intrinsically resistant to chemotherapy and nearly 50% of originally responsive patients develop chemoresistance during the course of their treatment (Perez et al, 1993). In addition, ovarian cancers may show significant differences in chemosensitivity, even if they share identical clinicopathological features.

An increasing body of evidence suggests that most anti-cancer agents commonly used in the treatment of several malignancies may induce tumour regression by apoptosis (Hickman, 1992; Carson and Ribeiro, 1993; Fisher, 1994; Thompson, 1995), a peculiar form of cell death with biochemical and morphological features distinct from those of necrosis (Majno and Joris, 1995).

The apoptotic process is modulated by some proto-oncogenes and tumour-suppressor genes (Kerr et al, 1994; Thompson, 1995). Among the latter, the p53 gene seems to have a crucial role in the execution of some forms of apoptosis (Yonish-Rouach et al, 1991; Shaw et al, 1992; Kerr et al, 1994). It has been shown *in vitro* that loss of p53

function, through mutations that interfere with apoptosis, facilitates the development of neoplastic clones (Symonds et al, 1994) resistant to different antineoplastic drugs (Lowe et al, 1993a) including CDDP (Nabeya et al, 1995). In addition, p53 mutations could exert a specific activation of the MDR1 (multiple drug resistance) gene promoter (Chin et al, 1992), which may result in a reduced intracellular concentration of various chemotherapeutic agents (Juliano and Ling, 1976).

p53 alterations are common genetic events in ovarian carcinomas (Kohler et al, 1993; Kupryjanczyk et al, 1993; Milner et al, 1993), but at the moment it is not clear whether they have a prognostic value (Bosari et al, 1993; Niwa et al, 1994; Kappes et al, 1995) and their role in chemoresistance has not been established.

The present study was undertaken to investigate the prognostic and predictive role of p53 alterations in ovarian cancer. To this end, we evaluated the status of the p53 gene by genetic analysis and the expression of the p53 protein by immunohistochemistry in a series of 68 ovarian carcinomas. The results were compared with well-known clinicopathological parameters of prognosis and with response to antineoplastic agents in a subset of 33 patients treated with CDDP-based chemotherapy.

## MATERIALS AND METHODS

### Patients and tissue samples

Sixty-eight patients with ovarian neoplasm, including 15 with low malignant potential (LMP) tumours (Hart, 1992) and 53 with invasive ovarian carcinoma, were analysed in this study.

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Tumour specimens were consecutively obtained at initial surgical resection and immediately frozen at  $-70^{\circ}\text{C}$ . To overcome the limitations due to the heterogeneity of ovarian cancer, adjacent sections of tumour were obtained in each case for DNA extraction and for immunoperoxidase staining. In each case, segments of normal fallopian tube or peripheral blood were collected as controls.

The carcinomas were histologically typed and graded according to the World Health Organization (Serov et al, 1973). Only epithelial ovarian malignancies were included in this study; among the invasive tumours there were 31 serous, ten endometrioid, three mucinous, three clear cell and six undifferentiated carcinomas. All LMP tumours were classified as serous. With respect to grade, there were 12 well-differentiated (G1), 14 moderately (G2) and 27 poorly differentiated tumours (G3).

Tumour stage was determined according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO) (Beahrs et al, 1988). For statistical analysis, patients with stage I and stage II disease were pooled into an early stage subgroup, whereas patients with stage III and IV disease were pooled into an advanced stage subgroup; 14 of the patients under study had stage I–II disease and 39 patients had stage III–IV disease.

The age range of these patients was 41–81 years, with a median of 59 years.

Forty-six patients with follow-up data ranging from 8 to 92 months' duration were analysed for progression-free length of survival. The evaluation of the clinical course of disease was based on clinical examination, chest radiography, abdominal–pelvic ultrasound and computerized tomography (CT) scan. After initial surgery, 33 patients with stage III–IV invasive ovarian cancer received six cycles of CDDP-based chemotherapy. The disease progressed or remained stable in four patients. The other 29 patients who achieved a clinical complete or partial response to chemotherapy underwent a second-look laparotomy. A pathological complete response to therapy was defined as the disappearance of all tumour deposits with negative peritoneal washing and negative multiple random biopsies.

### Detection of p53 alterations by SSCP

Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis (Orita et al, 1989) was performed to detect mutations in the exon 5–8 of the *p53* gene. Samples were subjected to amplification at each exon, including the exon–intron boundary. The technique was optimized as previously described (Spinardi et al, 1991). Routinely, 100 ng of genomic DNA was used in a 10- $\mu\text{l}$  PCR reaction containing 10 mM Tris-HCl (pH8.3), 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.01% (w/v) gelatine, 1.25 mM each of four dNTPs (Boehringer Mannheim Biochemica), 1 mM of each primer, 0.5  $\mu\text{l}$  of [ $\alpha$ - $^{32}\text{P}$ ]dCTP (3000 Ci  $\text{mmol}^{-1}$ , Amersham, Arlington, IL, USA) and 0.25 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). Four couples of specific intronic primers, whose sequences were deduced from Buchman et al (1988), were used:

#### Exon 5:

Sense: 5'-TGACTTTCAACTCTGTCTCCT  
Anti: 5'-TCAGTGAGGAATGAGAGGCC

#### Exon 6:

Sense: 5'-CTGGAGAGACGACAGGGCTG  
Anti: 5'-CCAFACACCCAGTTGCAAAC

#### Exon 7:

Sense: 5'-CTCGCGCACTGGCCTCATCTT  
Anti: 5'-TCAGCGGCAAGCAGAGGCTG

#### Exon 8:

Sense: 5'-GGACAGGTAGGACCTGATTCCTTAC  
Anti: 5'-TGCACCCTTGGTCTCCTCCAC

Thirty amplification cycles were performed under the following conditions: initial denaturation, 6 min at  $94^{\circ}\text{C}$ ; amplification, 1 min at  $94^{\circ}\text{C}$ , 1 min at  $58^{\circ}\text{C}$ , 2 min at  $72^{\circ}\text{C}$  for 30 cycles; elongation, 5 min at  $72^{\circ}\text{C}$ .

PCR products were electrophoretically separated by 6% non-denaturing polyacrylamide gel at 10 W for 12 h at  $20^{\circ}\text{C}$ . Genomic DNAs from tumour samples and corresponding normal ovarian tissues were examined by separate PCR–SSCP analysis to exclude the possibility of any polymorphism within the coding region. Upon complete migration, the gels were dried and subjected to autoradiography against a Kodak XAR-5 film at  $-80^{\circ}\text{C}$  with an intensifying screen. PCR was performed at least twice for each sample, and only the reproducible cases were taken.

### Immunohistochemical analysis

Immunostaining of the p53 protein was performed by the avidin–biotin peroxidase complex method. Briefly, 4–5  $\mu\text{m}$  frozen sections were cut, mounted on polylysine-coated slides, air dried and fixed in cold ( $4^{\circ}\text{C}$ ) acetone for 10 min before being subsequently incubated with: (1) monoclonal antibody 1801 (Ab-2; Oncogene Science, Vector, Burlingame, CA, USA) (diluted 1:100, overnight at  $4^{\circ}\text{C}$ ) which recognizes a denaturation-resistant epitope from amino acid 32 to 79 of the p53 protein; (2) a biotinylated horse anti-mouse IgG antibody (diluted 1:500, 30 min at room temperature); (3) avidin–peroxidase complexes (1 h). Finally, the slides were developed with 0.5% diaminobenzidine in 0.05 M Tris buffer pH 7.4 containing 0.5% hydrogen peroxide, rinsed in tap water, counterstained with 5% haematoxylin, dehydrated, cleared in xylene and mounted in permanent coverslipping medium. Positive controls were two known positive cases of human colon cancer. Negative controls were obtained by replacement of primary antiserum with Tris buffer.

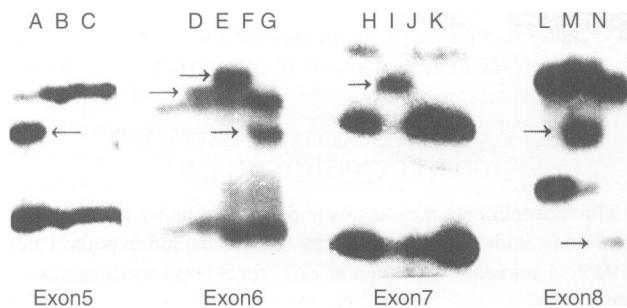
### Statistical analysis

Contingency tables were used to examine the relationship between aberrations of the *p53* gene and each of the following clinicopathological data: stage of disease, residual disease, grade of tumour differentiation, response to chemotherapy and progression-free overall survival. Statistical association was determined by  $\chi^2$  analysis. A *P*-value of less than 0.05 was considered to have statistical significance.

## RESULTS

### p53 gene mutations

In all the LMP tumours tested, no *p53* gene mutations were found. Electrophoretic mobility shifts, indicative of mutation of *p53*, were detected in 28 (53%) of 53 invasive carcinomas; 11 mutations were located in exon 5, six in exon 6, eight in exon 7 and three in exon 8 (Figure 1). A strong correlation ( $P=0.006$ ) was observed between *p53* gene mutations and FIGO stage; *p53* mutations were found in 3 (21%) of 14 tumours at stage I–II and in 25 (64%) of 39 cases at stage III–IV (Table 1). A higher frequency of

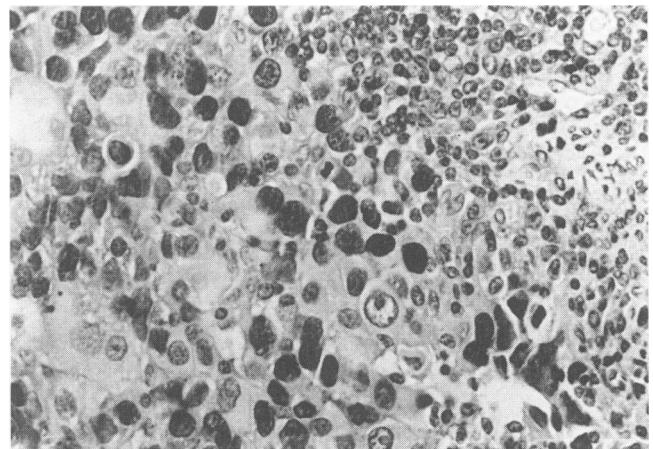


**Figure 1** PCR-SSCP analysis of exons 5–8 of the *p53* gene in 14 different cases (A–N) of ovarian carcinomas. The arrows indicate bands with mobility shift. Common polymorphisms of the *p53* gene were excluded by using DNA from normal tissue as comparison in each patient

*p53* mutations was detected in poorly differentiated tumours; of 28 tumours with *p53* gene alteration, two (7%) were well differentiated (G1), nine (32%) moderately differentiated (G2) and 17 (61%) poorly differentiated (G3). This difference was statistically significant ( $P=0.01$ ). No correlation was present between aberrations in the *p53* gene and the histological type of the tumours examined. A statistically significant association ( $P=0.05$ ) was also present between *p53* gene mutations and progression-free survival; of 13 patients with short (<18 months) or absent progression-free survival, ten (77%) had *p53* mutations.

#### Nuclear accumulation of the p53 protein

*p53*-immunoreactive cells were never observed in LMP tumours nor in normal ovarian tissues. In all positive cases, the immunoreactivity was nuclear and present in more than 10% of neoplastic cells (Figure 2). Twenty-seven (51%) of 53 invasive ovarian cancers showed a clear overexpression of the *p53* protein. A correlation



**Figure 2** Nuclear accumulation of the *p53* protein in a case of poorly differentiated ovarian carcinoma. Most of the neoplastic cells show a strong immunostaining (immunoperoxidase technique)

( $P=0.05$ ) was found between *p53* nuclear accumulation and stage of disease; 4 (15%) out of 27 cases that scored positive for *p53* overexpression were at stage I–II, whereas 23 (85%) were in advanced stages (III–IV). A significant association ( $P=0.01$ ) was also observed between nuclear accumulation of the *p53* protein and tumour differentiation; 2 (17%) out of 12 G1 tumours, 7 (50%) of 14 G2 tumours and 18 (67%) of 27 G3 tumours were *p53* positive. This difference was statistically significant ( $P=0.01$ ). *p53* nuclear accumulation was more frequently seen in patients with shorter progression-free survival; in the group of patients with shorter (<18 months) progression-free survival, 69% (9 out of 13 cases) had *p53* nuclear accumulation, whereas in the group of patients with longer (>18 months) progression-free survival a *p53* overexpression was observed in 42% (14 out of 33) of cases. This difference was not significant ( $P=0.1$ ).

**Table 1** *p53* status and main clinicopathological features in invasive ovarian cancer

Clinicopathological features	Molecular analysis (SSCP)		<i>P</i> -value	Immunohistochemical analysis		<i>P</i> -value
	<i>p53</i> positive	<i>p53</i> negative		<i>p53</i> positive	<i>p53</i> negative	
Mean age at diagnosis (years)	58	62	NS <sup>a</sup>	58	62	NS
FIGO stage						
Stage I/II	3 (21) <sup>b</sup>	11 (79)		4 (29)	10 (71)	
Stage III/IV	25 (64)	14 (36)	0.006	23 (59)	16 (41)	0.05
Differentiation						
well differentiated	2 (17)	10 (83)		2 (17)	10 (83)	
moderately differentiated	9 (64)	5 (36)	0.01	7 (50)	7 (50)	0.01
Poorly differentiated	17 (63)	10 (37)		18 (67)	9 (33)	
Response to chemotherapy						
Complete	1 (14)	6 (86)		1 (14)	6 (86)	
Partial	15 (68)	7 (32)	0.009	14 (64)	8 (36)	0.01
Absent	4 (100)	0		4 (100)	0	
Progression-free survival						
> 18 months	15 (45)	18 (55)		14 (42)	19 (58)	
<18 months	10 (77)	3 (23)	0.05	9 (69)	4 (31)	NS

<sup>a</sup>NS, not significant. <sup>b</sup>Numbers in parentheses are percentages.

**Table 2** Correlation of p53 status and chemosensitivity

Case no.	Stage	SSCP analysis <sup>a</sup>	IHC analysis <sup>b</sup>	SSCP+IHC	Response to chemotherapy <sup>c</sup>
1	III	+ (5)	+	+	C
2	III	-	-	-	C
3	III	-	-	-	C
4	III	-	-	-	C
5	III	-	-	-	C
6	III	-	-	-	C
7	III	-	-	-	C
8	III	+ (5)	+	+	P
9	III	+ (7)	+	+	P
10	III	+ (5)	+	+	P
11	III	+ (7)	+	+	P
12	III	+ (6)	+	+	P
13	III	+ (6)	+	+	P
14	IV	+ (5)	+	+	P
15	III	+ (7)	+	+	P
16	IV	+ (5)	+	+	P
17	III	+ (7)	+	+	P
18	IV	+ (6)	+	+	P
19	III	+ (6)	-	+	P
20	III	+ (6)	-	+	P
21	III	+ (5)	-	+	P
22	III	+ (5)	-	+	P
23	IV	-	+	+	P
24	III	-	+	+	P
25	III	-	+	+	P
26	III	-	-	-	P
27	III	-	-	-	P
28	III	-	-	-	P
29	IV	-	-	-	P
30	III	+ (6)	+	+	NR
31	IV	+ (7)	+	+	NR
32	III	+ (5)	+	+	NR
33	IV	+ (5)	+	+	NR

<sup>a</sup>Numbers in parentheses correspond to exon mutated. <sup>b</sup>IHC, immunohistochemistry. <sup>c</sup>C, complete; P, partial; NR, no response.

### Relationship between SSCP and immunohistochemical data

A strong correlation ( $P=0.001$ ) was observed between *p53* mutations detected by SSCP analysis and nuclear accumulation of the *p53* protein evaluated by immunohistochemistry. The immunohistochemical staining and the SSCP analysis gave concordant results in all the LMP tumours tested. In the 53 invasive carcinomas, corresponding results were obtained in 42 (79%) cases; *p53* gene mutation with nuclear accumulation of its protein product was seen in 22 cases, while in 20 cases there was absence of both mutation and overexpression. However, in 6 (21%) of 28 cases with a *p53* mutation, no nuclear accumulation was present and in 5 (20%) of 25 cases without mutation, an immunoreactivity for the *p53* protein was seen.

### p53 alterations and response to chemotherapy in invasive ovarian carcinomas

After initial cytoreductive surgery, 33 patients with advanced (stage III–IV) invasive ovarian carcinomas were treated with six cycles of CDDP-based chemotherapy. *p53* gene status and expression were evaluated in tumour samples obtained before therapy. Mutations of the *p53* gene were detected in 20 (61%) of 33 cases; nine mutations were located in exon 5, six in exon 6 and five in

exon 7 (Table 2). No mutations were observed in exon 8. Using immunohistochemistry, a nuclear accumulation of *p53* protein was found to be present in 19 (58%) of 33 cases. A significant association was observed between *p53* alterations and response to chemotherapy with both SSCP ( $P=0.009$ ) and immunohistochemical ( $P=0.01$ ) methods. However, a very strong correlation ( $P=0.001$ ) emerged by cumulating the results of the genetic and immunohistochemical analysis; *p53* aberrations were observed in only one (14%) of seven patients who achieved a pathological complete response, in 18 (82%) of 22 patients who had partial response and in all of the four patients who did not respond to chemotherapy. Because of the small number of patients with minimal residual disease (three cases), no significant relationship was detected between residual tumour volume and *p53* status or between residual tumour volume and chemosensitivity.

### DISCUSSION

Ovarian cancer is the gynaecological neoplasm with the most aggressive behaviour and worst prognosis. In about 70% of cases, ovarian cancer is diagnosed in advanced stage (Piver et al, 1991); this is presumably because of the paucity of specific, early symptoms and the lack of sensitive screening methods. In addition, despite the combined use of aggressive cytoreductive surgery and chemotherapy, long-term survival has not significantly improved in the last few years (Bookman and Bast, 1991).

Prognosis is currently based on clinical and histopathological factors (Friedlander and Dembo, 1991). However, the identification of new prognostic indicators could be of great value in the planning of individualized and potentially more effective treatments. A number of genetic alterations have been observed in ovarian cancer, but little is known about their prognostic role. *C-myc* amplification (Baker et al, 1990; Sasano et al, 1992) or *Ki-ras* mutations (Yaginuma et al, 1992) are involved in a very low percentage of cases. *C-erb-B2* gene amplification and/or overexpression has been demonstrated in 20–30% of ovarian malignant tumours (Kacinski et al, 1992; Fajac et al, 1995), but these alterations do not seem to be important in identifying subsets of patients with poor prognosis.

So far, the gene most frequently mutated in ovarian cancer (50–70% of cases) is *p53* (Marks et al, 1991; Mazars et al, 1991; Okamoto et al, 1991; Eccles et al, 1992). Such a high frequency of mutations suggests an important role for this gene in ovarian carcinogenesis. Although a consistent literature provides data indicating that the loss of *p53* function may have a prognostic value in different forms of human tumours, including lung (Marchetti et al, 1993), breast (Bosari et al, 1992; Bergh et al, 1995) and prostatic malignancies (Bauer et al., 1995), it is not clear whether *p53* alterations may have a similar value in ovarian cancer. In our series of ovarian carcinomas, *p53* gene aberrations were significantly associated with advanced stages of disease ( $P=0.006$  and  $P=0.05$  for *p53* mutations and *p53* protein overexpression respectively), and they were more prevalent among poorly differentiated tumours ( $P=0.01$ ). In previous studies, no correlation between *p53* alterations and stage was reported (Marks et al, 1991; Hartmann et al, 1994; Kappes et al, 1995). Many variables may account for this difference, including the method used in the study, the quality of neoplastic samples (fixed or frozen) and the origin of material (primary tumour or recurrence). It is interesting to note that in the series of Marks et al (1991), only 15 out of 107 cancers analysed were early stage, and in six of these cases tissue was obtained at the

time of recurrence. On the other hand, Kappes et al (1995) examined different types of ovarian malignant tumours, including epithelial and non-epithelial forms, primary and secondary (metastatic) neoplasms. The authors reported that the presence of p53 aberrations was not associated with stage of disease. However, they observed a clear correlation between the frequency of p53 mutations and malignant potential of the tumours; somatic mutations were very frequent in tumours of high grade and with peritoneal spread. Concerning the relationship between p53 abnormalities and tumour differentiation, most authors (Bosari et al, 1993; Milner et al, 1993) have found that the presence of p53 aberrations is significantly related to higher tumour grade. In agreement with recently published data (Bosari et al, 1993; Levesque et al, 1995), we have also observed that patients with p53 mutations have a significantly shorter progression-free survival than patients with p53-negative tumours ( $P=0.05$ ). In addition, the observation that benign and borderline tumours, as well as most of the early-stage carcinomas, were negative for p53 abnormalities while advanced-stage ovarian carcinomas frequently showed p53 alterations strongly suggests that abnormalities in the p53 gene may be associated with acquisition of aggressive behaviour and metastatic phenotype. In regard to this, Kupryjanczyk et al (1993) reported a statistically significant association between p53 protein accumulation in stage III disease and small primary tumour size at diagnosis, suggesting that p53 abnormal proteins could accelerate the metastatic spread.

In our panel of 33 patients with known response to chemotherapy, we found p53 mutations in 73% (19 out of 26) and p53 protein overexpression in 69% (18 out of 26) of the cases with partial or absent response to chemotherapy. On the other hand, only 14% of the patients with complete response to chemotherapy showed p53 aberrations. A very strong correlation ( $P=0.001$ ) emerged by cumulating the results of the genetic and immunohistochemical analyses. These results can be explained by considering the role of p53 in mediating the apoptotic process induced by antineoplastic drugs. Recent studies in Chinese hamster ovary cells (Barry et al, 1990; Eastman, 1990) and in a preleukaemia cell line (Miyashita and Reed, 1993) have demonstrated that the cytotoxic effect of CDDP, one of the more effective and more commonly used drugs in the treatment of ovarian cancer (Gately and Howell, 1993), is based on induction of apoptosis. The role of apoptosis in cell killing by CDDP is also demonstrated by flow cytometric methods (Ormerod et al, 1994). A link between the wild-type p53 gene and execution of apoptosis following DNA damage by anticancer drugs (Lowe et al, 1993a) or ionizing radiation (Lowe et al, 1993b) has also been observed in different cell lines (O Connor et al, 1993; Nabeya et al, 1995) and in murine model systems (Lowe et al, 1994) with defective p53. In addition, by using p53-null mice, it has been shown that p53 inactivation causes a decrease in the level of apoptosis and rapid tumour growth (Symmonds et al, 1994). Recently, it has been reported in mouse p53-expressing tumours that acquired mutations in the p53 gene are associated with both treatment resistance and relapse (Lowe et al, 1994). To the best of our knowledge, the present study is the first to demonstrate in vivo an association between p53 gene alterations and chemosensitivity in human ovarian cancer. Similar results have recently been obtained using an in vitro assay in a series of patients with primary untreated breast carcinomas (Koechli et al, 1994).

When our manuscript was ready to be submitted, another study (Righetti et al, 1996) presented experimental data very similar to the results reported in the present study. The authors analysed 32

untreated patients with ovarian carcinoma, and they found a correlation between p53 alterations and response to cisplatin-based chemotherapy.

In conclusion, taken together our data strongly suggest that in epithelial ovarian malignancies tumours showing p53 aberrations are significantly less sensitive to chemotherapy and more aggressive than those with functional p53. Thus, a routine analysis of this gene could have profound implications for the treatment of ovarian cancer.

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