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P53 Gene Status in Patients with Advanced Serous Epithelial Ovarian Cancer in Relation to Response to Paclitaxel- plus Platinum-based Chemotherapy and Long-term Clinical Outcome

ANGIOLO GADDUCCI[1], CLAUDIO DI CRISTOFANO[2], MICHELE ZAVAGLIA[2], LAURA GIUSTI[2], MICHELE MENICAGLI[2], STEFANIA COSIO[1], ANTONIO G. NACCARATO[2], ANDREA R. GENAZZANI[1], GENEROSO BEVILACQUA[2] and ANDREA O. CAVAZZANA[2]

[1]*Department of Procreative Medicine, Division of Gynecology and Obstetrics and*
[2]*Department of Oncology, Division of Surgical, Molecular and Ultrastructural Pathology, University of Pisa, Pisa, Italy*

Abstract. *Background: The aim of this retrospective study was to assess whether p53 gene status has any predictive or prognostic relevance in patients with advanced, poorly-differentiated serous epithelial ovarian cancer treated with paclitaxel- plus platinum-based chemotherapy. Materials and Methods: The study was conducted on 46 patients who underwent surgery followed by paclitaxel- plus carboplatin-based chemotherapy. The tumor tissue samples were analyzed for p53 gene mutations. The median follow-up of survivors was 50.3 months. Results: Twenty-three patients (50%) showed p53 mutations at exons 5 to 9. Sixteen (34.8%) patients had a polymorphism at codon 72 in exon 4 (SNP codon 72): 10 were Pro/Pro homozygous and 6 Pro/Arg heterozygous. Four polymorphic patients had a second mutation at exons 5 to 9. An inverse correlation was evidenced between the SNP codon 72 and mutations at exons 5 to 9, with the latter more frequently found in wild-type (Arg/Arg) codon 72 (19/30 versus 4/16, 63.3% versus 25.0%; p=0.03) cases. A clear trend for a higher response rate and longer progression-free and overall survival was observed in wild-type p53 and Pro/Pro polymorphic patients as compared to patients with mutant p53. Conclusion: The addition of paclitaxel to carboplatin does not appear to overcome the negative predictive and prognostic significance of p53 gene mutations in serous ovarian cancer. Nevertheless, the comprehensive analysis of p53 genotype, including the SNP codon 72, warrants further investigation in order to envisage individual responsiveness to cancer therapy.*

Correspondence to: Angiolo Gadducci, Department of Procreative Medicine, Division of Gynecology and Obstetrics, University of Pisa, Via Roma 56, Pisa, 56127, Italy. Tel: 39 50 992609, Fax: 39 50 553410, e-mail: a.gadducci@obgyn.med.unipi.it

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Epithelial ovarian cancer belongs to the 5 leading causes of tumor mortality in women in developed countries (1). Approximately 70% of epithelial ovarian cancers are detected at an advanced stage, mainly due to the lack of reliable screening methods. Cytoreductive surgery followed by platinum-based chemotherapy has long been considered the standard therapeutic strategy. In the last decade, the combination of paclitaxel plus cisplatin has been shown to obtain a significantly longer progression-free survival and overall survival (2, 3). Furthermore, paclitaxel- plus carboplatin- based regimens appeared to have equivalent activity to paclitaxel- plus cisplatin- based regimens but improved toxicity profile (4, 5). The combination of paclitaxel plus carboplatin is now widely accepted as the standard front-line chemotherapy for advanced epithelial ovarian cancers (6-9) .

Experimental studies on ovarian cancer cell lines and primary tumors have shown that induction of apoptosis in response to cytotoxic drugs, such as cisplatin, cyclophosphamide or paclitaxel, represents the main mechanism of tumor cell death (10). The wild-type *p53* gene product is involved in the cellular response to a number of cytotoxic insults, through cell cycle regulation, DNA repair and activation of apoptotic pathways (11). *P53* mutations and/or p53 protein overexpression have been detected in 20-79% of epithelial ovarian cancers, and are more frequently observed in advanced than in early stages of the disease (12-29). *In vitro* experiments in platinum-resistant ovarian cancer cell lines pointed to the involvement of mutant p53 in the failure of cisplatin-induced apoptosis (30-33). Furthermore, transfection of the wild-type p53 *via* adenovirus significantly sensitized the human ovarian p53 mutant A2780/CP tumor cell line to cisplatin cytotoxicity (33). *In vivo* studies showed a significant correlation between p53 status and response to cisplatin- or carboplatin-based chemotherapy in ovarian cancer patients.

Table I. *PCR primers.*

4F/1 5'-AGGACCTGGTCCTCTGAC-3'	**4R**/1 5'-CTGGGAAGGGACAGAAGA-3'
4F/2 5'-CCCTGCACCAGCCCCCTCCT-3'	**4R**/2 5'-CCTAAGGGTGAAGAGGAATCCCA-3'
5F 5'-TGACTTTCAACTCTGTCTCCT-3'	**5R** 5'-TCAGTGAGGAATCAGAGGCC-3'
6F 5'-CTGGAGAGACGACAGGGCTGG-3'	**6R** 5'-CCAGAGACCCCAGTTGCAAAC-3'
7F 5'-AAGGCGCACTGGCCTCATCTT-3'	**7R** 5'-CGCCGGAAATGTGATGAGAG-3'
8F 5'-TGGTTGGGAGTAGATGGAGCC-3'	**8R** 5'-CACCGCTTCTTGTCCTGCTT-3'
9F 5'-GTGGAGGAGACCAAGGGTGCA-3'	**9R** 5'-AGGTAAAACAGTCAAGAAGAA-3'

Patients with p53-mutated tumors experienced a lower complete response rate than those with p53 wild-type tumors (16-18, 22, 23, 28, 34, 35). While the loss of p53 function is now widely accepted to represent one of the major mechanisms of platinum chemo-resistance, the role of p53 status as a prognostic factor is still matter of discussion. In a number of studies, p53 status did not correlate with survival (12, 14, 19, 21, 24, 26), whereas other authors reported poorer clinical outcome in patients with p53 alterations (15, 17, 20, 25, 28, 29, 36, 37).

Epithelial ovarian cancers comprise a broad spectrum of malignancies, ranging from serous to endometrioid, mucinous, transitional, clear cell and undifferentiated tumor types. These histotypes have been recently associated with distinct molecular profiles (38, 39), making it reasonable to conceive that the different molecular pathways may strongly affect the response to different drugs.

In the present study, a homogenous series of tumors belonging to the most frequent histotype of epithelial ovarian cancer was analyzed. Our aim was to assess the predictive and prognostic value of the *p53* gene status in patients with advanced, poorly-differentiated (G_3) serous epithelial ovarian cancer, who received first-line paclitaxel-plus carboplatin-based chemotherapy.

Materials and Methods

Patients. Forty-six consecutive cases of FIGO stage IIc-IV, serous G_3 epithelial ovarian cancer were retrieved from the records of the Division of Surgical, Molecular and Ultrastructural Pathology, Department of Oncology, University of Pisa (Italy). Representative tumor tissue blocks from primary tumors were selected for molecular analyses and tissue microarray (TMA) preparation.

All patients underwent primary cytoreductive surgery followed by first-line chemotherapy consisting of paclitaxel (175 mg/m^2 3-h infusion) plus carboplatin (Area under curve [AUC] 5-6) for 6 cycles at 3-week intervals at the Division of Gynecology and Obstetrics, Department of Procreative Medicine, University of Pisa between January 1996 and November 2003. The evaluation of the clinical course of disease was based on clinical examination, serum CA–125 assay, chest X-ray, abdominal-pelvic ultrasound and computed tomography scan. Additional investigations were performed when appropriate. After the sixth cycle of chemotherapy, patients with no evidence of disease at clinical, serologic, sonographic and radiologic examinations were defined as being in clinically complete response. Three to 5 weeks after the end of chemotherapy, a second-look surgery was usually proposed to clinically complete responders, mostly to patients enrolled in clinical trials. A pathological complete response at second-look surgery was defined as the disappearance of all macroscopic tumor deposits with negative peritoneal washing and negative multiple random biopsies. All patients with clinically or surgically detectable persistence disease, as well as some pathlogically complete responders received additional chemotherapy.

All patients were observed until death or until December 2004. The median follow-up of survivors was 50.3 months (range, 13 to 106 months).

Molecular analysis. The *p53* gene status was analyzed on formalin-fixed, paraffin-embedded tumor specimens. Representative tumor tissue sections (tumor area >80%) were cut and placed directly into a sterile microfuge tube. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen cat. N° 51304). All procedures were performed according to manifacturer's protocols.

In 4 cases, due to the low proportion of tumor cells, the specimens were subjected to laser capture microdissection (Leica AS LDM system). The dissected tumor cells (about 7,000 cells) were placed directly in 60 μl of DNA extraction buffer (100 mM Tris-HCl, pH 8; 1 mM EDTA; 1% Tween-20; 200-300 mg/ml Proteinase K) and incubated at 37°C for 12-16 hours. The reaction was heat terminated (95°C for 10 min).

Polymerase chain reaction (PCR) reactions were performed in 30 μl final volume, containing 2 μl of DNA, 2 mM dNTP (Eurobio), 250 ng/μl of each primer (MWG Biotech), 1.5 mM $MgCl_2$, 1 x PCR Gold buffer and 1U AmpliTaq Gold (PE Biosystems, Foster City, CA, USA).

The *p53* status was determined by direct sequencing from exon 4 to 9. PCR primers used are reported in Table I.

The expected fragment lengths for amplification products were as follows: 243 bp (4/1), 227 bp (4/2), 290 bp (5), 206 bp (6), 283 bp (7), 240 bp (8), and 227 bp (9).

The PCR reactions were performed using a 9700 GenAmp PCR System (Applera), with the following conditions: initial denaturation, 7 min at 95°C; amplification, 45 s at 94°C, 45 s at 58°C, 1 min at 72°C (40 cycles).

The PCR products were purified using Multi Screen PCR Plates (Millipore) and were sequenced in 10 μl final volume, using Big Dye Terminator kit v3.1 (Applera) and 2.5 pmol of primers. Sequencing products were run on an ABI PRISM 3100 Genetic Analyzer (Applera) and analyzed with GeneScan software Sequencing Analysis vers.3.7 (ABI PRISM).

Immunohistochemical analysis. Thirty-six samples were used for the immunohistochemical analysis of *p53* gene product expression in

Table II. *Relationship between p53 status at the molecular level and its expression.*

Immunostaining p53	Mutant p53	SNP codon 72 Pro/Pro p53	Wild-type p53	Overall
Positive	13 (52.0%)	7 (28.0%)	5 (20%)	25
Negative	5 (45.4%)	1 (9.1%)	5 (45.4%)	11

Table III. *P53 gene status and complete response rate to chemotherapy.*

p53 gene status	Patients	Complete responders	
	N	N	%
Mutant	23	14	60.8
Wild-type	11	10	90.0
SNP codon 72 Pro/Pro	8	7	87.5
SNP codon 72 Pro/Arg	4	1	25.0

TMA. Core tissue biopsies (1 mm diameter) were taken from representative regions of paraffin-embedded ovarian tumors (donor block) and arrayed into a new recipient paraffin block (45 mm x 20 mm), using an ATA-100 Chemicon International System. In order to minimize the influence of tumor heterogeneity, 3 different core biopsies for each donor block were retrieved together with paired normal tissue as internal control. The arrays contained 50 tissue cylinders, including 10 primary tumors and 10 corresponding controls. Antigen retrieval was accomplished by microwaving (360 watt) the slides for 5 min (3 cicles) in 1 mmol/L citrate buffer pH 7. Primary antibodies were omitted in negative controls. Commercially available prediluted monoclonal p53 antibody (clone Bp53-11, Ventana) at 1:10 diluition was employed, using an automated system (NEXES, Ventana). Tumors were scored as p53-positive when >10% of tumor nuclei were stained in at least one of the tumor spots.

Statistical analysis. Rates of complete response were compared to *p53* gene status using the Pearson χ^2 test (or the two-tailed Fisher's exact test when appropriate). The cumulative probability of progression-free survival and overall survival from the time of initial surgery was estimated by the product-limit method. The log-rank test was used to correlate progression-free survival and overall survival curves to *p53* status.

Results

The median age of patients was 55 years (range, 41 to 73 years). According to the FIGO classification, the tumor stage was IIc in 5 patients, III in 35 patients and IV in 6 patients. After primary cytoreductive surgery, 22 patients had residual disease ≤1 cm and 24 patients had a larger residual tumor. Ascites was detected in 25 patients.

Molecular analysis. Twenty-three (50.0%) out of 46 patients showed mutant *p53*. In detail, 3 mutations were detected in exon 5, 4 in exon 6, 6 in exon 7, 8 in exon 8 and one mutation was found in exon 9. Only one patient showed a double missense mutation (exons 5 and 6). Missense mutations (20/23) were the most common, with transitions (C>A) more frequent (14/20) than trasversions (6/20). Two deletions and 2 insertions were also found. Codons 7 and 8 were most frequently affected, accounting for approximately two-thirds of the mutations.

A polymorphism at codon 72 in exon 4 (SNP codon 72) was observed in 16 (34.8%) patients. Ten were Pro/Pro homozygous and the remaining 6 were Pro/Arg heterozygous. Four polymorphic patients (2 Pro/Pro and 2 Pro/Arg) also showed an a second mutation at exons 5 to 9. For statistical purposes, they were included within the *p53* mutant group. An inverse correlation was evidenced between SNP codon 72 and mutations at exons 5 to 9, with the latter more frequently found in wild-type (Arg/Arg) codon 72 (19/30 *versus* 4/16, 63.3% *versus* 25.0%, $p=0.03$) patients.

Immunohistochemical analysis. Twenty-five (69.4%) out of 36 samples submitted to immunohistochemical analysis scored positive for p53. No significant correlation was found between p53 expression and *p53* gene status (Table II) ($p=0.21$). Among the 25 patients with positive p53 immunostaining, 13 (52%) had p53 mutations at exons 5 to 9 and 5 (20.0%) had wild-type p53. The remaining 7 (28.0%) cases were polymorphic at codon 72. Seven out of 8 Pro/Pro and 5 out 10 wild-type p53 samples stained positive.

Clinical analysis. After the sixth cycle of chemotherapy, 11 patients achieved a pathologically complete response at second-look surgery, 21 patients obtained a clinically complete response but were not submitted to second-look surgery and 14 patients had clinically or surgically detectable persistent disease. Therefore, taking into consideration the best assessed response, a complete (either clinical or pathologic) response was observed in 32 patients.

Complete response rates were higher in patients with wild-type p53 as compared to patients with mutant p53 (90.0% *versus* 60.8%, $p=0.11$) (Table III). Among the 12 p53 wild-type polymorphic patients, the homozygous Pro/Pro patients experienced higher complete response rates than heterozygous ones (87.5% *versus* 25.0%, $p=0.07$). Complete response rates were obtained in 18 (72.0%) out of 25 patients with positive immunostaining for p53 compared to 6 (54.6%) out of 11 patients with negative p53 expression ($p=0.44$).

For statistical purposes, the patients with Pro/Arg polymorphism were not included in progression-free

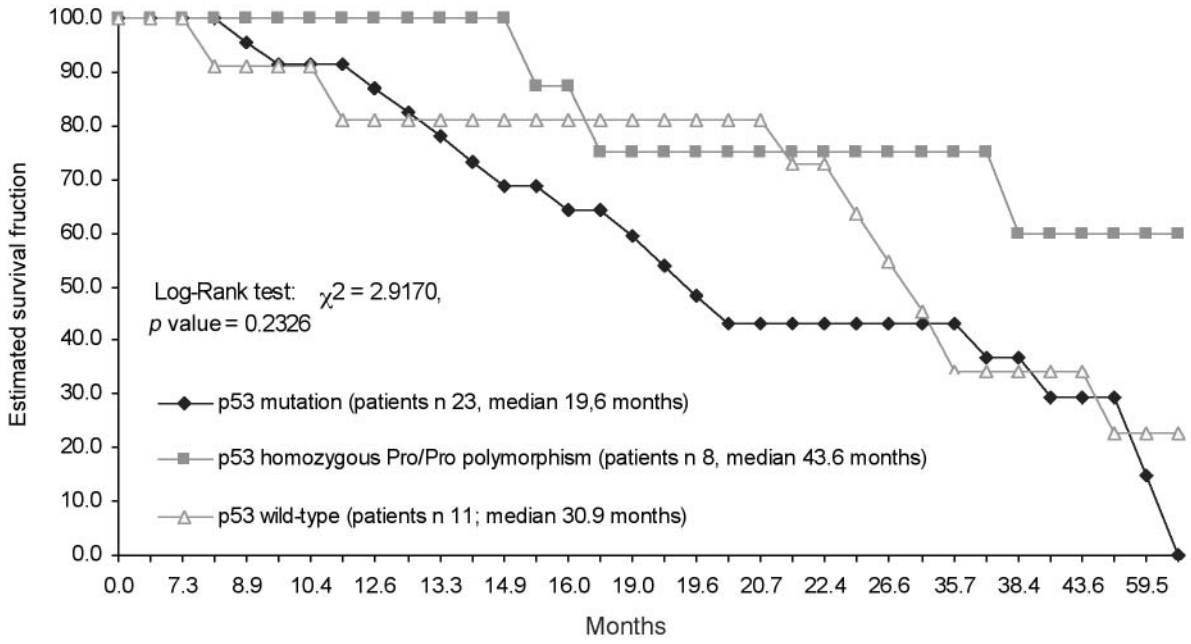

Figure 1. *Progression-free survival in patients with advanced serous poorly-differentiated epithelial ovarian cancer by p53 gene status.*

survival and overall survival analysis, due to the limited number of cases. Patients with wild-type p53 as well as those with homozygous Pro/Pro polymorphism had a better progression-free survival (Figure 1) and overall survival (Figure 2) when compared to patients with mutant p53, although the differences were not statistically significant.

No correlation was found between p53 immunoreactivity and progression-free or overall survival (data not shown).

Discussion

Epithelial ovarian cancers represent the overwhelming majority of ovarian malignancies, with the serous histotype being the most common. In the last decade, complete response rates as well as short-term survival have significantly improved, but long-term clinical outcome remains unsatisfactory. *P53* gene inactivation has been found to confer resistance to cisplatin and other DNA-damaging agents (11). Conversely, recent clinical studies reported that patients with mutant p53 tumors were responsive to paclitaxel- plus platinum-based chemotherapy (18, 19, 21-23). The mechanism of action of taxanes consists of alterations in microtubule function and the presence of a functional *p53* gene does not seem to be required for apoptotic cell death induction by antimicrotubule agents (22). Furthermore, pharmacological studies support the notion of increased sensitivity to taxanes by mutant p53 cells, due to the accumulation of treated cells in the G_2-M phase (40). Lavarino *et al.* (18) found that all but one of the 10 ovarian cancer patients who showed p53 accumulation by immunocytochemistry achieved a pathologically or clinically complete response to paclitaxel- plus carboplatin-based chemotherapy. Seven out of the 10 p53-positive cases were missense mutations. Smith-Sorensen *et al.* (19) detected p53 mutations in 73% of tumor samples from 45 ovarian cancer patients randomized to receive paclitaxel-plus-cisplatin or cyclophosphamide-plus-cisplatin. Despite the lack of information on tumor histotype, it was found that, among p53 mutated patients, relapse-free survival was significantly longer for the paclitaxel-plus-cisplatin group compared with the cyclophosphamide-plus-cisplatin group. Moreover, p53 status was found to be prognostically irrelevant for the patients treated with paclitaxel-based regimens. In a retrospective investigation on 43 patients with advanced ovarian cancer treated with paclitaxel-based chemotherapy, Laframbroise *et al.* (23) showed that p53 status was neither predictive of chemoresistance nor prognostic of disease-free and overall survival. A multicentric Italian study (22) assessed p53 status by genetic analysis of exons 5 through 8 in tumor specimens collected at the time of initial surgery from 48 advanced ovarian cancer patients who subsequently received paclitaxel-plus platinum- based chemotherapy. Twenty-five (86.0%) out of 29 patients with mutant p53 responded to therapy as opposed to only 9 (47.0%) out of 19 patients with wild-type p53. Actuarial overall survival analysis revealed no significant difference between mutant and wild-type p53 cases.

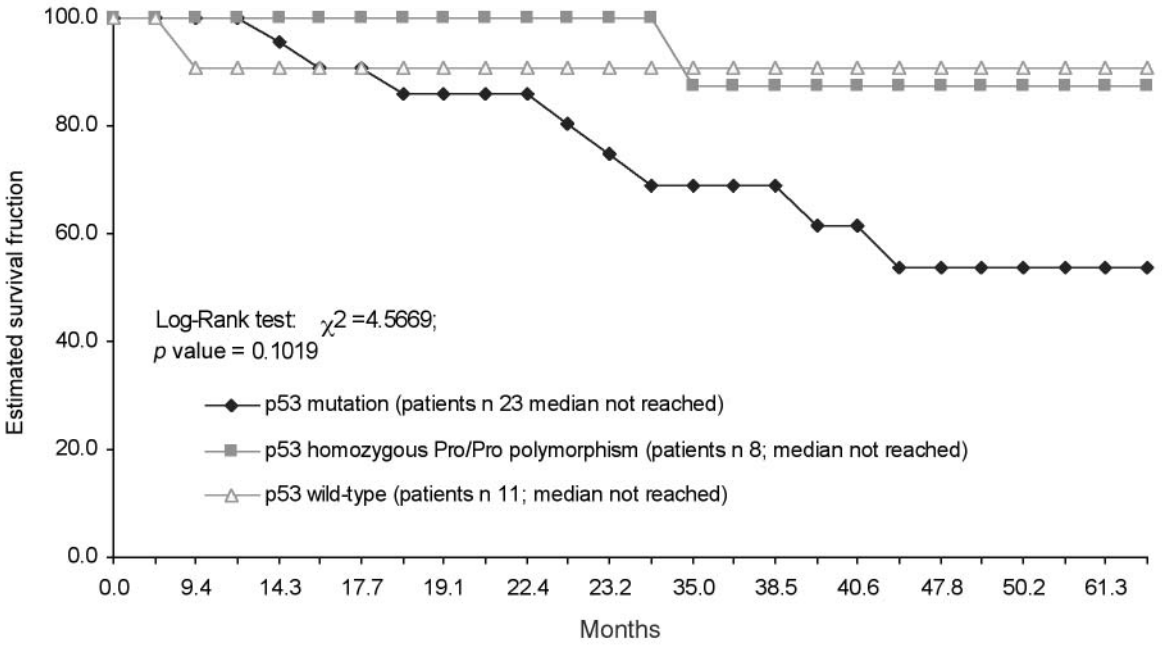

Figure 2. *Overall survival in patients with advanced serous poorly-differentiated epithelial ovarian cancer by p53 gene status.*

To date no information is available for serous carcinoma *per se*. Here, we presented a series of 46 patients with advanced, G_3 serous epithelial ovarian cancer. Twenty-three (50.0%) had *p53* mutations at exons 5 to 9 and 16 (34.8%) showed SNP codon 72. Of these latter, 10 were Pro/Pro homozygous and 6 were Pro/Arg heterozygous. Only 4 polymorphic patients showed a second mutation at exons 5 to 9, and an inverse correlation was evidenced between the SNP codon 72 and mutations at exons 5 to 9, with the latter more frequently found in wild-type codon 72 (63.3% *versus* 25.0%, $p=0.03$) patients. Complete response rates were appreciably higher in patients with wild-type p53 compared to patients with mutant p53 (90.0% *versus* 60.8%). Among the polymorphic patients with no associated mutations in exons 5 to 9, Pro/Pro homozygous patients experienced a higher complete response rate than Pro/Arg heterozygous ones (87.5% *versus* 25.0%). Recently, it has been shown that the SNP codon 72 modulates the response to chemotherapy both *in vitro* and *in vivo* (41). This effect may be explained by the capability for some tumor-derived p53 mutants to bind and inactivate *p73*, a *p53*-related gene, which also induces apoptosis (42). The amount of p73 protein in cells has been shown to be increased by cisplatin (43). Binding of p53 mutants to p73 appears to be influenced by whether codon 72 encodes arginine or proline. In fact, the ability of p53 to bind p73, to neutralize p73-induced apoptosis and to transform cells in cooperation with EJ-Ras was enhanced when codon 72 encoded arginine (42). Bergamaschi *et al.* (44) showed that head and neck squamous cell carcinomas characterized by the Arg polymorphism had a response rate to cisplatin-based chemo-radiotherapy lower than those with a Pro polymorfism. In our series, the Pro/Pro patients showed a trend to a higher response rate, longer progression-free survival and longer overall survival as compared to p53 mutants. This finding suggests a role for the SNP codon 72 in the response of advanced epithelial ovarian cancer to paclitaxel- plus carboplatin based-chemotherapy; further studies enrolling a larger number of patients are warranted to elucidate its function.

Patients with mutant *p53* gene showed a lower response rate and worse clinical outcome when compared to patients with either wild-type or homozygous polymorphic *p53* gene, thus confirming the negative predictive and prognostic role of a mutated *p53* gene in human malignancies. The addition of paclitaxel to carboplatin does not seem as effective in the treatment of p53-mutated ovarian carcinomas as reported in previous studies (19, 22, 23). Discrepancies with the literature may be ascribed, in part, to the different histological features of the tumors analyzed in different series. The percentage of serous carcinomas included in previously reported studies ranges from 56% to 77% (22, 23). Considering the different molecular pathways involved in ovarian cancerogenesis (38, 39), histological heterogeneity may constitute a severe bias in drawing definitive results.

As regards the immunohistochemical studies, the detection of p53 accumulation does not appear a reliable

method to predict either response to treatment or clinical outcome. Moreover, immunohistochemical results may be misleading in judging *p53* gene status; in our hands there was no correlation between p53 status at the molecular level and its expression.

In conclusion, *p53* gene status, as determined at the molecular level, remains a prominent prognostic tool to predict the clinical outcome of patients affected by serous epithelial ovarian cancer treated with paclitaxel- plus carboplatin-based regimens. In particular, the comprehensive analysis of the p53 genotype, including the SNP codon 72, appears to represent a valuable tool in conceiving individual responsiveness to cancer therapy.

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