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Drug Resistance in Ovarian Cancer – the Role of p53

Russel PETTY,[1] Alan EVANS,[2] Iain DUNCAN,[1] Christian KURBACHER,[3] Ian CREE[4]

[1]Department of Obstetrics and Gynaecology, and [2]Department of Pathology, Ninewells Hospital and Medical School, Dundee, Scotland; [3]Labor für Chemosensitivitatestungen Universitats – Frauenklinik, University of Cologne Medical Center; Cologne, Germany; [4]Department of Pathology, Institute of Ophthalmology, University College London, London, England

The aims were to determine the importance of p53 and bcl-2 expression on the response to chemotherapy with alkylating agents in patients with ovarian cancer. We have followed the response to chemotherapy in a series of 59 patients with ovarian adenocarcinoma designated as p53 and bcl-2 positive or negative by immunocytochemistry. Of these cases, 50 received either cisplatin + treosulfan or treosulfan alone. Immunocytochemistry for p53 was positive in 28/59 tumors. Patients were grouped according to their response to chemotherapy (stable or progressive disease) assessed at 6, 12, and 18 months. There was increasing divergence of p53+ and p53- tumors over time. Of those which were p53+, 25% showed progression at 6 months, 80% at 12 months and 89% progression at 18 months. In contrast, 23%, 50%, and 67% of p53- tumors showed progression at 6 , 12 and 18 months respectively. For bcl-2, in 23/55 positive tumors there was progression in 35%, 78% and 94% compared with 25%, 57% and 59% in bcl-2 negative tumors at 6, 12 and 18 months respectively. Those tumors which were bcl-2 and p53 negative were most likely to progress, while those which were bcl-2 and p53 positive had the best prognosis. These differences did not translate into increased overall survival with minimum follow-up of 12 months. This data lends support to our suggestion that despite initially increased susceptibility to alkylating agents, enhanced genomic instability due to p53 inactivation may render tumors more likely to develop resistance to chemotherapy over time. This effect may be altered by bcl-2 function, lack of which will lead to a good response to chemotherapy as the tumor's ability to undergo apoptosis will not be compromised. (Pathology Oncology Research Vol 4, No 2, 97–102, 1998)

Keywords: ovary, adenocarcinoma, p53, immunostaining, bcl-2, chemotherapy

Introduction

Multiple abnormalities of genes controlling growth and differentiation underly oncogenesis. In most tumors, mutation of a tumor suppressor gene is implicated as an early event since cells with these defects are more likely to acquire the number of mutations necessary for eventual tumor formation. p53 is probably the most studied tumor suppressor gene and is mutated or inactivated in a large proportion of cancers. Wild type p53 has a number of known cellular functions including its ability to respond to DNA damage by mediating cell cycle arrest in G1/S and in some situations, apoptosis.[21,34]

Since alkylating agents act by promoting DNA damage, p53 status is likely to influence the response to tumors to such agents. Previous in vitro/ex vivo investigations have shown increased sensitivity of solid tumor cells with mutant/inactivated p53,[11,12,31,37] while hematogenous cells show enhanced resistance, probably because p53-mediated apoptosis occurs more readily in these tumors.[4,8,24,25,35,40] Ovarian cell lines transfected with mutant p53 gene constructs may also show enhanced sensitivity.[2] Differences in the effect of p53 on the response to chemotherapy are probably due at least in part to the existence of different thresholds for triggering apoptosis between different cell types.[15] However, there is also evidence that the apoptosis pathway

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Correspondence: Dr. Ian CREE, Department of Pathology, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL; Tel: 0171-608-6808, Fax: 0171-608-6862; e-mail: i.cree@ucl.ac.uk

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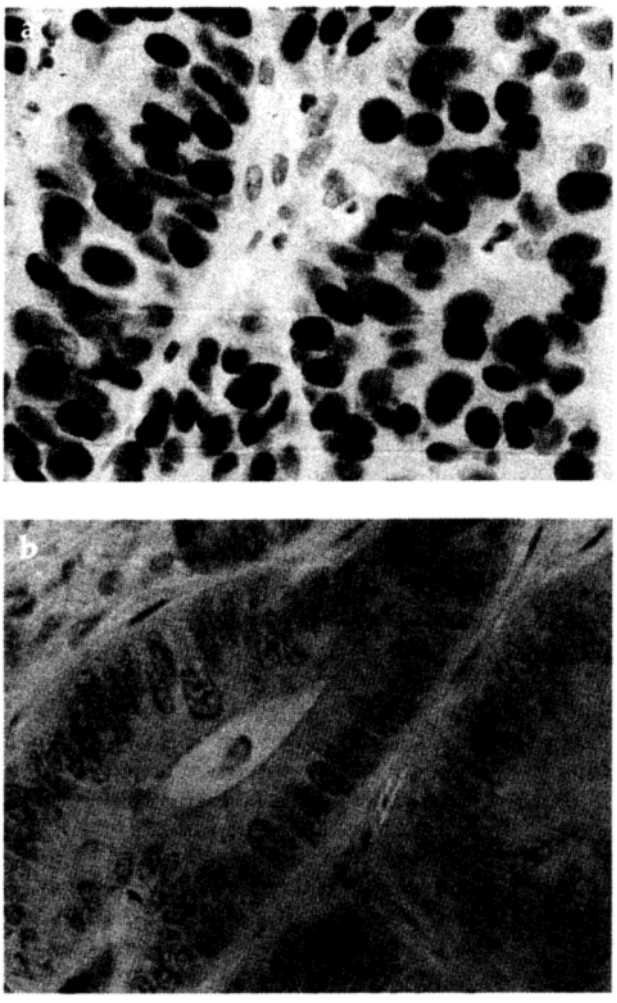

Figure 1. *(a) A p53 positive tumor showing heavy nuclear staining in virtually all neoplastic nuclei. Stromal cells are negative. (b) A p53 negative tumor showing only occasional positively stained cells. Final magnification x1,720*

may be compromised in many epithelial carcinomas, including those of breast and ovary. Protection against p53-mediated apoptosis following exposure to alkylating agents has been noted in ovarian cancer cell lines.[16] Some evidence points to bcl-2 overexpression in these tumors as a likely explanation,[13,15] although the reason for this overexpression does not appear to be mutation of bcl2 itself. Since increased bcl-2 expression is associated with protection against apoptosis, p53-mediated initiation of apoptosis would be ineffective.[15] Tumor cells would be released from the G1-S checkpoint control normally mediated by p53 to progress through the cell cycle with considerable DNA damage, leading to non-survivable division or mutation. While the former would result in increased responsiveness in affected cells, the latter is likely to of greater importance to the ability of the tumor to evade chemotherapy and produce early recurrence.[31]

Most solid tumors have a high recurrence rate following chemotherapy, and we wondered if the genomic instability engendered by p53 mutation/inactivation might lead to more rapid development of chemoresistance or selection of chemoresistant clones within the tumor over a time span not tested in short term cell culture assays. Few tumors are treated with alkylating agents alone, but primary ovarian carcinoma treatment is often based on a combination of two alkylating agents, one of which is usually a platinum compound. Since p53 mutation is common in ovarian cancer, we have examined a series of primary ovarian carcinomas to determine whether there is a relationship between the rate of recurrence following chemotherapy and p53/bcl-2 status.

Materials and Methods

Patients

A series of 100 consecutive ovarian cancer patients was selected from the files of the Tayside gynaecological cancer registry from January 1992 - November 1994. 81 cases received chemotherapy and in 59 of these, histological material was available. The mean age was 63 years (range 30 to 87 years). Twelve patients had stage I disease, three stage II, 29 stage III and 15 stage IV. Histologically, 10 tumors were described as adenocarcinoma (not otherwise specified), 25 endometrioid, 4 mucinous, 19 serous, and one clear cell. Thirty patients were treated with cisplatin + treosulfan, 20 with single agent treosulfan , three with cisplatin alone, one with carboplatin alone, two with a combination of treosulfan and carboplatin, and 1 with melphalan as a single agent. The remaining two patients received megestrol acetate, a steroid compound, and were excluded from further analysis.

Immunostaining

Formalin fixed, paraffin-embedded blocks of tissue were cut at 5 μm thickness to provide material for immunostaining. Sections were dewaxed by immersion in Histoclear and a series of alcohols. After washing in distilled water, sections immersed in 10 mM citric acid monohydrate buffer, pH 6.0, were microwaved (750W) for 5x5 min, ensuring that they were completely immersed in buffer throughout each 5 min incubation period. Following the last microwave treatment, the sections were cooled to room temperature and transferred to phosphate buffered saline (PBS, pH 7.4) and washed for 5 min. All subsequent incubations were performed at room temperature (20°C) and dilutions performed in PBS. Non-specific binding was blocked by 20 min incubation with a 1:20 dilution of normal rabbit serum (Scottish Antibody Production Unit, SAPU) and the sections then incubated

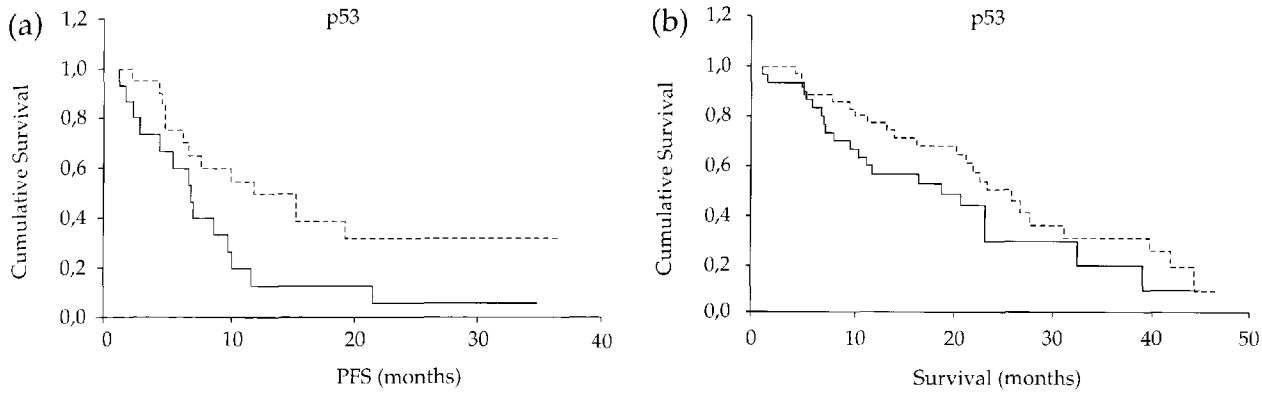

Figure 2. *(a) Kaplan-Meier curve for p53+ (———) and p53- (– – –) tumors showing the increased progression-free survival (PFS) of p53- tumors (p<0.04, log rank test). (b) Kaplan-Meier curve for p53+ (———) and p53- (– – –) tumors showing little effect of p53 on survival (NS, log rank test).*

for 60 min in a 1:500 dilution of polyclonal sheep anti-p53 antibody (SAPU). After washing for 5 min in PBS, the sections were incubated for 30 min in a 1:9 dilution of biotinylated anti-sheep rabbit immunoglobulin (Sigma Chemical Co. Ltd., Poole, Dorset). After washing in PBS for 5 min, the sections were incubated for 20 min in 1:25 streptavidin-peroxidase (Biogenix). Following a further wash in PBS, the sections were incubated for 5 min with diaminobenzidine/nickel chromogen (Vector Labs) and washed again prior to counterstaining with weak haematoxylin. For bcl-2 immunohistochemisty, a monoclonal antibody from Dako (High Wycombe, Bucks, UK) was demonstrated using a biotin-avidin kit purchased from the same company. After dehydration the sections were mounted in DPX and examined by direct microscopy. Coded sections were assessed for p53 and bcl-2 positivity (*Figure 1*) independently of the known response to chemotherapy.

Assessment of response to chemotherapy

Patients were assessed as having stable or progressive disease at 6, 12 and 18 months after the start of their first chemotherapy regimen. Those patients who died from their disease were included with the progressive group. Once classified as progressive, patients did not revert to a stable classification even if they responded to subsequent courses of chemotherapy. In addition, the date of death of each patient was recorded with a minimum follow-up period of two years.

Data analysis

The results of p53 immunostaining and response to chemotherapy were collected in a database (Access ver 1.1, Microsoft, USA) and analysed using SPSS for Windows.

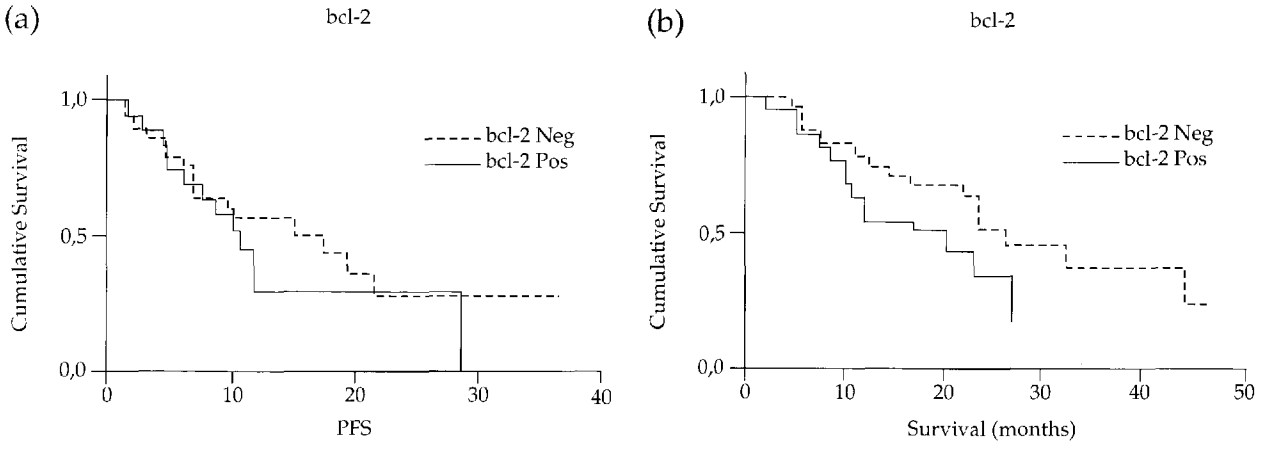

Figure 3. *(a) Kaplan-Meier curve for bcl-2 positive (———) and bcl-2 negative (– – –) tumors showing effect on PFS. (b) Kaplan-Meier curve for bcl-2 positive (———) and bcl-2 negative (– – –) tumors showing no statistically significant effect on survival.*

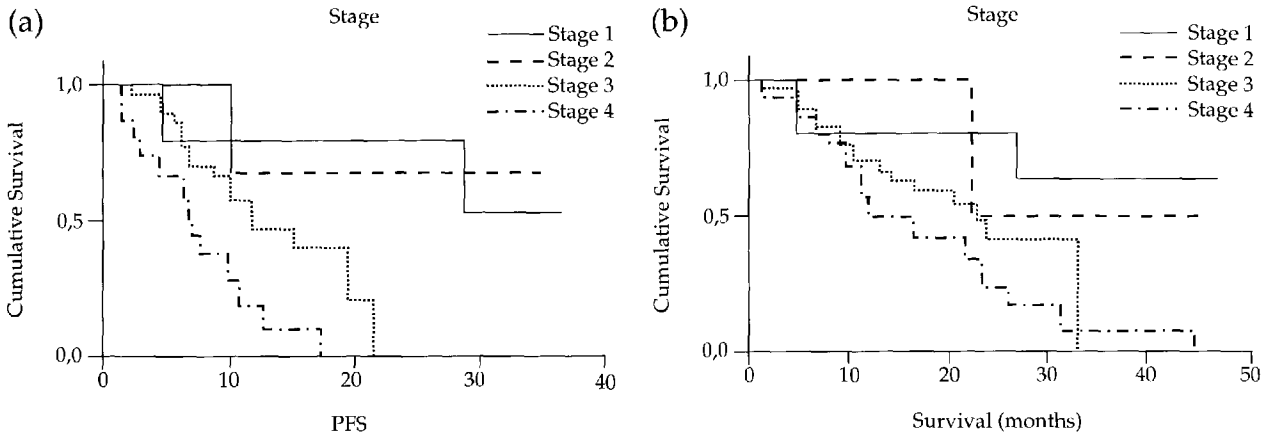

Figure 4. The effect of stage on (a) PFS and (b) survival of ovarian tumors (both p<0.03 log rank test). Those with advanced (stage 3/4 disease) recur and die much more rapidly that those with limited (stage 1/2 disease).

Results

Two patterns of p53 immunostaining were defined. p53+ tumors were those in which nearly all the tumor cells present showed positive nuclear staining, while p53- tumors showed no nuclear staining (*Figure 1*). Intermediate staining of some but not all tumor cells was not observed in this series. Of the 50 patients treated with cisplatin + treosulfan or treosulfan alone, 28 were p53+ and the remainder p53-. The p53+ tumors had a significantly higher chance of early recurrence in comparison with p53- tumors (*Figure 2a*, p<0.04). There was little difference at 6 months (25% p53+ v. 23% p53-, NS), but p53+ tumors were significantly more likely to recur at 12 months (80% p53+ v. 50% p53-, p<0.03). This difference persisted at 18 months after diagnosis (88% p53+ v. 67% p53-, p<0.01), but the majority of tumors had recurred. There was no effect of p53 on survival (figure 2b).

In general, tumors were either completely negative for bcl-2 or showed patchy positivity (*Figure 1*). Seven of the 23 positive tumors showed more widespread staining, but this small subgroup was not analysed separately. The bcl-2 positive tumors had a worse prognosis than the bcl-2 negative tumors (*Figure 3*). Although there was little difference at 6 months (35% bcl-2+ v. 25% bcl2-, NS), at 12 months 78% of the bcl-2 positive tumors had relapsed in comparison with 57% of the bcl-2 negative tumors (NS) and at 18 months there were 94% relapses in the bcl-2 positive tumor with only 59% in the negative group (p < 007). However, despite an obvious trend in survival in Kaplan-Meier graphs of bcl-2+ and bcl-2- tumors, this did not reach statistical significance (p<0.091, NS).

The effect of stage on PFS and survival (*Figure 4*) is much greater than p53 status. 4/13 stage 1/2 tumors and 22/44 stage 3/4 tumors were p53+, but this difference was not statistically significant. The p53 effect on PFS persisted if just those with stage 3/4 disease were considered (p<0.05), but there was no statistical difference within the small number of stage 1/2 tumors included in the study. No statistical differences were noted for bcl-2 in stage 1/2 or 3/4 patients when these were considered separately.

Discussion

Our results show that despite an identical response to chemotherapy at six months, p53+ tumors with mutant or inactivated p53 recur more quickly than p53- tumors. However it should be noted that the difference is not sufficiently large to be clinically useful as an indicator of prognosis. Similar results have been shown by many other studies.[10,22,30,38,39] Despite occasional reports to the contrary,[1,6,14,33] most studies agree that p53 status alone does not have a large influence on overall survival. Initial enthusiasm for immunostaining p53 as a marker for malignancy and as a guide to treatment appears to have been largely misplaced.

The results of this and other studies are consistent with the hypothesis that increased genomic instability due to p53 abnormality leads to an increased ability to evolve chemoresistance over time. This probably involves multiple mechanisms with both enhanced gene amplification and deletion following p53 mutation.[23] In ovarian cancer, our study suggests that the key period of p53 influence seems to be between 6 and 12 months. This fits well with our suggestion that genomic instability mediated by p53 mutation/inactivation may render tumors more likely to develop resistance to chemotherapy over time.[31]

The effect of p53 mutation of tumor sensitivity to alkylating agents will vary according to the cells' susceptibility to apoptosis. Those cells which upregulate bcl-2 will survive apoptotic insults and would therefore be expected to have a worse prognosis if this is in fact the main form

of cell death occurring in response to such chemotherapy.[27] However, in our series bcl-2 appeared to have a late effect on prognosis and length as well as size of future prognostic studies of bcl-2 will be important. Eliopoulos et al[9] have shown chemoresistant cases to have increased bcl-2 expression. While it certainly seems from this and other studies that bcl-2 positivity may an adverse prognostic factor, others have shown it to be associated with a good prognosis[5,17,18] emphasising the heterogeneity and complexity of bcl-2 effects on differing molecular backgrounds within tumor cells. However, all studies show that oncogene effects on either overall survival or PFS are much less than clinical stage. There were too few patients in this series to allow statistical comparison of p53 and bcl2 status together, but there were roughly equal numbers of p53+/bcl2+, p53+/bcl2-, p53-/bcl2+, p53-/bcl2- tumors (13, 14, 10, and 18 out of 55 respectively) suggesting that there is no correlation between the activity of these two oncogene products in ovarian carcinoma.

Enthusiasm for determining the molecular basis of cancer chemosensitivity and resistance has not always been matched by an appreciation of the complexity of the intracellular and tissue-based mechanisms involved.[3,7,26,28,31,32] It is clear from this work and an increasing body of other research that p53 is just one of many factors affecting alkylating agent sensitivity. No one factor predicts tumor behaviour or survival.[3] In ovarian cancer, the contribution of bcl-2 and other oncogenes which modulate cellular susceptibility to apoptosis may be of considerable importance in response to chemotherapy,[9] despite the apparent lack of effect on prognosis in this study. There is already evidence that bcl-2 expression is a favourable prognostic factor in breast cancer[20] and in ovarian cancer bcl-2 expression has been shown to correlate inversely with the apoptotic count within the tumor.[41] It is possible that the predictive value of bcl-2 immunohistochemistry could be improved by measurement of bax expression as the bax/bcl-2 ratio has recently been shown to have prognostic significance in one study.[27] However, such statistical analyses of large series of cases are a long way from methods useful for individualised chemotherapy.[3] Further developments should include clinical trials comparing different treatments based on the molecular make-up of the tumor. Recent success with anti-bcl-2 therapy in melanoma[19] could be applied to ovarian cancer.

Will therapeutic correction of p53 inactivation help? Although the proportion of stage 1/2 tumors showing p53 positivity was not significantly lower than stage 3/4 tumors, larger studies[7] have found evidence that p53 abnormalities occur late in ovarian oncogenesis. If this association reflects biological behaviour secondary to the loss of p53 function, then correction of p53 might be clinically useful.[36] If lack of p53 function provides enhanced chemosensitivity in the first days following alkylating agent administration, followed by an enhanced likelihood of resistance, correction of p53 function should take place following standard alkylating agent chemotherapy. This hypothesis is testable and we hope that future clinical trials will address this issue.

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