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p53 And related proteins in epithelial ovarian cancer

P.S. Sengupta [a], A.T. McGown [b], V. Bajaj [c], F. Blackhall [a], R. Swindell [d], M. Bromley [e], J.H. Shanks [f], T. Ward [b], C.H. Buckley [g], K. Reynolds [h], R.J. Slade [h], G.C. Jayson [a,*]

[a] *Cancer Research Campaign Department of Medical Oncology, Christie Hospital National Health Trust, Withington, Manchester M20 4BX, UK*
[b] *Section of Drug Development and Imaging, Paterson Institute for Cancer Research, Withington, Manchester M20 9BX, UK*
[c] *Medical School, Manchester University, Oxford Road, Manchester M13 9PT, UK*
[d] *Department of Medical Statistics, Christie Hospital National Health Trust, Withington, Manchester M20 4BX, UK*
[e] *Section of Histology, Paterson Institute for Cancer Research, Withington, Manchester M20 9BX, UK*
[f] *Department of Histopathology, Christie Hospital National Health Trust, Withington, Manchester M20 4BX, UK*
[g] *Department of Histopathology, St Mary's Hospital National Health Trust, Whitworth Park, Manchester M13 0JH, UK*
[h] *Department of Gynaecology Oncology, Christie Hospital National Health Trust, Withington, Manchester M20 4BX, UK*

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Abstract

We conducted a retrospective immunohistochemical evaluation of the prognostic significance of the expression of p53 and the related proteins Bax, Bcl-2, growth arrest and DNA damage (Gadd45), murine double minute 2 (Mdm2) and $p21^{WAF1/CIP1}$ in chemonaïve tumours taken from 66 patients with ovarian cancer. Ki-67 expression (a marker of cell proliferation) was also evaluated immunohistochemically, while apoptosis within malignant cells was determined with the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay. The expression of each of the following proteins was significantly associated in the tumours ($P < 0.05$ unless otherwise stated): Bax with Bcl-2 ($P < 0.01$); Bax with Mdm2; $p21^{WAF1/CIP1}$ with Gadd45 ($P < 0.01$); $p21^{WAF1/CIP1}$ with p53; p53 with Mdm2. Univariate analysis showed that expression of p53, Bax, bulk residual disease and International Federation of Gynecology and Obstetricians (FIGO) stage were all strongly correlated with response to chemotherapy ($P < 0.01$). Similarly, the FIGO stage and Ki-67 expression ($P < 0.01$), as well as pathological subtype and bulk residual disease ($P < 0.05$), were prognostic factors for disease progression. The FIGO stage and Ki-67 expression were significant prognostic factors for overall survival ($P < 0.01$), with Gadd45 expression and pathological subtype also significant ($P < 0.05$) in a univariate analysis. Multivariate analysis for response to chemotherapy showed that expression of p53, Bax and FIGO stage were all independent prognostic factors ($P < 0.01$). The FIGO stage was the most important independent prognostic factor for progression and survival on multivariate analysis ($P < 0.01$). However, Ki-67 expression was also an independent prognostic factor for disease progression ($P < 0.05$) and approached significance for survival ($P = 0.055$). Taken together, these data suggest that determination of Ki-67 expression could supplement established prognostic factors.

Keywords: Ovarian cancer; p53; Stage; Ki-67

1. Introduction

Epithelial ovarian cancer is the leading cause of death amongst the gynaecological cancers with an annual incidence of 6000 in the UK [1] and 27 000 in the USA [2]. Survival is poor because the disease has an insidious onset and many women present with advanced disease which is incurable by surgery and chemotherapy. Although chemotherapy prolongs survival [3], most patients with advanced disease die from chemoresistant progressive disease.

Chemoresistance may be related to defects in the apoptotic cell death mechanism that is dependent on a cascade of proteins centered on the *TP53* gene product, which is produced in response to DNA damage, and which, in turn, leads to apoptosis or cell cycle arrest. The importance of p53 in human malignancies was highlighted by the detection of abnormal p53 in more than 50% of human cancers [4–6]. In addition, families with inherited defects in *TP53* (Li-Fraumeni families) are susceptible to a wide range of cancers at an early age

* Corresponding author. Tel.: +44-161-446-3740; fax: +44-161-446-3299.
E-mail address: gordonjayson@christie-tr.nwest.nhs.uk (G.C. Jayson).

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[7]. Abnormal p53 protein is resistant to degradation and has a prolonged half-life which allows its detection by immunohistochemical staining [8]. This has a 96% sensitivity for missense mutations in ovarian cancer [9] and consequently can be used as a simple screening technique to identify p53 dysfunction [10].

The role of p53 in ovarian cancer is contentious, as there are a number of contradictory studies. Several studies have identified p53 protein expression, detected by immunohistochemistry, as an adverse prognostic factor for survival in human ovarian cancer [11–18]. Other studies have suggested that alterations in p53 expression in ovarian cancer affect sensitivity to chemotherapy [19,20]. In contrast, there are a number of studies that suggest that p53 expression has no prognostic value in epithelial ovarian cancer [21–26].

Proteins of the Bcl-2 family are critical regulators of the apoptotic pathway. Certain members of the family promote apoptosis (e.g. Bax, Bad, Bcl-X_S), while others have an anti-apoptotic function (Bcl-2, Bcl-X_L) [27]. The ratio of pro- and anti-apoptotic members, such as Bax and Bcl-2 is critical in the inhibition or induction of apoptosis [28]. The *TP53* gene product induces the expression of Bax and thus apoptosis [29]. Expression of Bcl-2 has been correlated with improved survival in ovarian cancer [14,15,30,31]. In contrast, Bax expression has been associated with unfavourable prognosis in ovarian cancer [31], and recent data revealed an association between Bax protein expression, improved response to chemotherapy and prolonged disease-free survival [32].

The cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1}$ is a downstream effector of p53 that is produced after DNA damage [33]. $p21^{WAF1/CIP1}$ inhibits the activity of several CDK–cyclin complexes [34,35] and also inhibits DNA replication through its interaction with proliferating cell nuclear antigen (PCNA), which is involved in DNA replication and repair [36,37]. Two studies found no correlation between expression of $p21^{WAF1/CIP1}$ and p53 in ovarian carcinoma [38,39], but did not examine the association in the context of established prognostic factors. However, a recent study detected an inverse relationship between expression of $p21^{WAF1/CIP1}$ and p53 [40]. The same study found that low $p21^{WAF1/CIP1}$ expression predicted poor overall survival on univariate, but not multivariate analysis, although patients with $p21^{WAF1/CIP1}$ positive/p53 negative tumours had a worse overall and recurrence-free survival.

The *Gadd45* (growth arrest and DNA damage inducible) gene product is a downstream effector of p53, induced following genotoxic stress and resulting in cell cycle arrest by a CDK-independent mechanism, providing wild-type p53 is present [41]. There have been no studies of the relationship between Gadd45 and ovarian cancer.

Murine double minute 2 (Mdm2) [42] represses *TP53* gene transcription and mediates the degradation of p53. Nevertheless, overexpression of Mdm2 is rare in ovarian cancers, irrespective of p53 mutation status [43].

The proliferation index has been correlated with prognosis and other clinicopathological features in a number of human malignancies [44–46]. The tumour proliferative fraction of ovarian carcinomas has been investigated immunohistochemically by means of antibodies recognising the nuclear antigen Ki-67 expressed in proliferating cells. Expression of the Ki-67 proliferation marker, which detects all phases of the cell cycle except G_0, is known to predict disease outcome in many human malignancies [46]. A number of studies on ovarian cancer have reported an association between a high proliferation index and reduced overall survival [47–52] or reduced disease-free survival [47,53]. However, Hartmann and colleagues [54] found improved survival for patients with ovarian tumours manifesting a high proliferation index. Multivariate analysis [53] revealed a significant relationship between high Ki-67 immunostaining in ovarian neoplasms and disease-free survival. Anttila and associates [55] also found that high proliferation was associated with poor prognosis for ovarian cancer on both univariate and multivariate analyses. However, Röhlke and associates [18] found that Ki-67 expression in ovarian neoplasms was not associated with overall survival.

A high apoptotic index has been found to be a poor prognostic indicator in a number of malignancies. However, on multivariate analysis, apoptotic index was not an independent prognostic factor for ovarian cancer [56].

We conducted a retrospective immunohistochemical evaluation of the prognostic significance of the expression of p53 and the related cell cycle proteins Bax, Bcl-2, Gadd45, Mdm2 and $p21^{WAF1/CIP1}$ in chemonaïve tumours taken from 66 patients with ovarian cancer. Ki-67 expression (a marker of cell proliferation) was also evaluated immunohistochemically, while apoptosis within malignant cells was determined through the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay. There have been no previous studies of this breadth in the p53 family for ovarian cancer. In addition, none have taken established prognostic factors into account for the analysis of such a wide range of biological factors.

2. Patients and methods

2.1. Patient selection

All the patients received chemotherapy at the Christie Cancer Centre. Tumour material was investigated from 66 formalin-fixed specimens. Postchemotherapy

material was available from 21 of these patients. The surgery was performed by a number of gynaecologists based at district general hospitals throughout the Northwest of England. Standard prognostic factors were evaluated for each patient including age at diagnosis, International Federation of Gynaecologists and Obstetricians (FIGO) stage [57], volume of residual disease following initial surgery, histological subtype, tumour grade, Karnofsky performance status (KPS) and response to the chemotherapy. The diagnosis of epithelial ovarian cancer and histological subtype [58] for each case was confirmed by a centralised pathology review before the study commenced. Only tumours acceptable as epithelial ovarian primaries were included in the series. Tumours were graded according to World Health Organization (WHO) criteria [59]. For the purposes of analysis, the population was separated into the histopathological subtypes serous, mucinous, endometrioid, clear cell, malignant Brenner tumour and other tumours (mixed and adenocarcinoma not otherwise specified).

Surgical and pathological findings, and postoperative abdomino-pelvic computer tomography (CT) scans were used to determine the FIGO stage for the ovarian cancer and the residual disease after the initial surgery. Persistence of tumour masses of less than or equal to 2.0 cm diameter after surgery was defined as minimal residual disease, whereas the presence of masses greater than 2.0 cm diameter was defined as bulk residual disease. The KPS recorded prior to chemotherapy was used for the study. Comparison between measurable malignant lesions on pre- and postchemotherapy abdomino-pelvic CT scans following chemotherapy were used to assess response to this treatment, and standard definitions of radiological response to chemotherapy were utilised (WHO, 1979). The patients were treated with platinum-based chemotherapy.

The overall survival time was calculated from the date of initial laparotomy to the date of death, or to the date the patient was last seen. Progression-free survival (PFS) was calculated from the date of registration to the date of documented disease progression as assessed by radiologically measurable disease on abdomino-pelvic CT scan or chest radiograph.

For univariate and multivariate statistical analysis, patients with FIGO stage I and II disease were grouped together, while those with stage III and stage IV were evaluated separately. Similarly, patients with KPS 30–70% were grouped together, while patients with KPS 80% and 90% were considered separately.

2.2. Immunohistochemistry

Parallel 4 μm serial sections cut from paraffin-embedded tumour blocks were mounted on Superfrost glass slides, dewaxed and rehydrated in a graded alcohol series, followed by microwave antigen target retrieval [60]. Immunostaining was performed using the rabbit or mouse DAKO EnVision™ + System, Peroxidase (DAB) Kit (DAKO Corporation, Carpinteria, CA, USA). The sections were incubated for 35 min with the following primary antibodies at the dilutions specified: rabbit polyclonal anti-Bax antibody (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:450; mouse monoclonal anti-Bcl-2 antibody (Clone 124) (DAKO) diluted 1:40; mouse monoclonal anti-Gadd45 antibody (C-4) (Santa Cruz Biotechnology) diluted 1:100; mouse monoclonal anti-$p21^{WAF1/CIP1}$ antibody WAF-1 (Ab-1) (Clone EA10) (Oncogene Research Products, Cambridge, MA, USA) diluted 1:40; mouse monoclonal anti-p53 antibody (DO-1) (Santa Cruz Biotechnology) diluted 1:200. For Mdm2 protein staining, two antibodies were used to control for the low expression in human epithelial ovarian cancer: mouse monoclonal anti-Mdm2 antibody (Clone SMP14) (DAKO) diluted 1:50; mouse monoclonal anti-Mdm2 antibody Mdm2 (Ab-1) (Clone IF2) (Oncogene Research Products) diluted 1:20. For statistical analysis, the Mdm2 antibody that showed the greater numbers of positive staining sections was used. The presence of proliferating malignant cells in each tissue section was confirmed by staining tissue sections for Ki-67 protein expression with monoclonal mouse anti-Ki-67 antibody (Clone 7B11) (Zymed Laboratories, Inc., San Francisco, CA, USA) diluted 1:100. Each stained section and the controls were examined with light microscopy by two independent observers who were unaware of the clinicopathological data and the results of scoring for each section with each of the other primary antibodies used. Each section was examined for positive staining in the cytoplasm and nuclei of the malignant cells, the absolute numbers of malignant cells stained, as well as the pattern and 'intensity' of the staining pattern in the sections. Tissues with defined expression or absence of expression of a particular antigen were used as positive and negative controls, respectively.

2.3. TUNEL assay for apoptosis

The presence of fragmented DNA from apoptotic malignant cells in each section was detected utilising the TUNEL assay, with a method similar to that previously described [56]. In the current study, the TUNEL assay was performed on formalin-fixed, paraffin-embedded tumour sections, using the Apoptosis Detection System, Fluorescein kit (Promega Corporation, Madison, WI, USA). Each tissue section was examined by two independent observers using fluorescence microscopy for the presence of localised bright green immunofluorescence denoting the presence of apoptotic cells. The morphology of the cells seen was also checked to ensure that they appeared apoptotic.

2.4. Statistical analysis

The χ^2 test for contingency tables or the Fisher's exact test were performed to determine the association between each biological factor and standard prognostic factors. The Wilcoxon Matched Pairs Signed Rank test was used to compare between the pre- and post-chemotherapy samples. Univariate graphs for PFS and overall survival were calculated using the method of Kaplan and Meier. Multivariate Cox proportional hazard regression analysis was performed on progression-free survival and overall survival. Univariate and multivariate analysis by logistic regression was used to assess response to chemotherapy. *P* values less than 0.05 were regarded as significant.

3. Results

3.1. Patient characteristics

The clinicopathological characteristics of the patients are shown in Table 1. None of the tumours considered for this study were of the mucinous subtype, although they had not been excluded from the study. Platinum-based chemotherapy was given following initial surgery to all but one patient (FIGO stage Ic; KP 90%) following initial surgery. 3 (5%) patients had no evaluable residual disease following initial surgery. Of the remaining patients, 46 (70%) responded to chemotherapy: 14 (21%) had a complete radiological response to chemotherapy and 32 (49%) had a partial response. 17 patients had no response to treatment: 5 (8%) had stable disease and 12 (18%) had progressive disease following chemotherapy. Follow-up data were available for 63 patients and the median follow-up for this group was 25 months (range: 9–125 months). The remaining 3 patients were lost to follow-up, and they had bulk residual disease with progressive disease on CT scan evaluation after completion of initial chemotherapy. Overall survival time for the 63 patients ranged from 6 to 76 months, with a median overall survival time of 29 months. During the time of follow-up, 27 patients were still alive with 36 deaths recorded. The median time to progression in the entire group of patients was 12 months (range: 1–75 months). The 5-year survival for patients diagnosed with FIGO stage I/II disease was 71%; for stage III it was 28%; for stage IV it was 11%.

Table 1
Clinicopathological characteristics of patients ($n = 66$)

Characteristic	*n* (%)
Median age	
55.5 years (range: 29–78 years)	66 –
Histopathology	
Serous	32 (48)
Endometrioid	12 (18)
Mucinous	0
Clear cell	6 (9)
Malignant Brenner tumour	2 (3)
Mixed; adenocarcinoma NOS	14 (21)
WHO tumour grade	
I: Well differentiated	6 (9)
II: Moderately differentiated	16 (24)
III: Poorly differentiated/undifferentiated	44 (67)
FIGO stage	
I	3 (5)
II	6 (9)
III	39 (59)
IV	18 (27)
Residual disease	
Nil	3 (5)
Minimal (0–2 cm)	14 (21)
Bulk (> 2 cm)	49 (74)
Karnofsky Performance status (KPS)	
90%	23 (35)
80%	15 (23)
30–70%	21 (32)
Unknown	7 (11)

NOS, not otherwise specified; WHO, World Health Organization; FIGO, International Federation of Gynaecologists and Obstetricians.

3.2. Scoring of stained sections

The slides were examined by two independent observers and scored for positive staining of the malignant areas of the sections by each antibody. Statistical analysis did not detect any differences in expression of each factor when classified as weak, moderate or strong. Hence, with the exception of staining for Bax, all sections were scored as either negative or positive.

Staining for Ki-67 was heterogeneous and restricted to the nucleus, and the sections were graded as positive if 10% or more of the cancer cells were stained (Fig. 1a). Similarly, staining for apoptosis was heterogeneous and sections graded as positive if they contained 10% apoptotic malignant cells.

All the sections stained positively for Bax expression. However, the pattern of staining in the malignant tumour cells was found to be either cytoplasmic or a combination of nuclear and cytoplasmic. Where nuclear staining was present in an individual tumour cell, the cell cytoplasm also showed positive staining. Sections with positive staining of the tumour cell population showed either cytoplasmic staining alone throughout the section, or combined nuclear and cytoplasmic staining throughout the section. Staining in the sections showing cytoplasmic staining alone was less intense (weak) than staining in the sections showing combined nuclear and cytoplasmic staining (strong). Hence, positive staining for Bax was divided into weak (cytoplasmic) (Fig. 1b) when cytoplasmic staining only was

detected, or strong (nuclear) when combined cytoplasmic and nuclear staining were present (Fig. 1c).

In sections that stained positively for Bcl-2 (Fig. 1d) or Gadd45 (Fig. 1e), the pattern of staining of malignant cells was cytoplasmic only, whereas in sections that stained positive for $p21^{WAF1/CIP1}$ (Fig. 1f) or p53 (Fig. 1g) the pattern of staining of malignant cells was nuclear. The pattern of staining of these cells throughout the sections was heterogeneous. However, positive samples were identified when at least 10% of the malignant cell population expressed the antigen. Mdm2 staining was heterogeneous and limited to the nucleus for both antibodies used (Fig. 1h). Due to the low expression of Mdm2, sections were described as positive

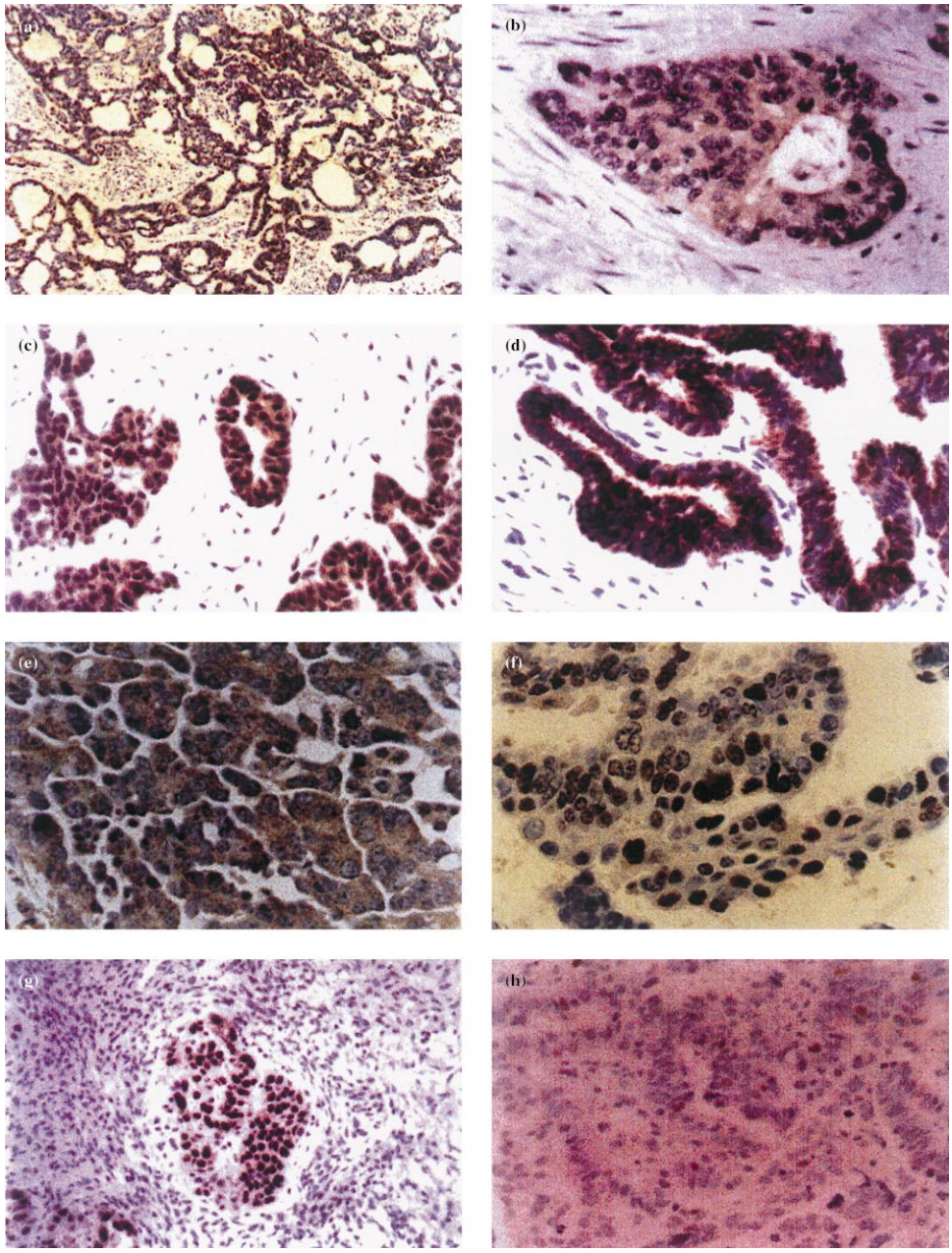

Fig. 1. (a) Positive Ki-67 immunostaining ($\geqslant$10% of cancer cells) in a moderately differentiated endometrioid ovarian carcinoma; (b) weak (cytoplasmic) Bax immunostaining in a poorly differentiated ovarian carcinoma; (c) strong (combined nuclear and cytoplasmic) Bax immunostaining in a moderately differentiated endometrioid ovarian carcinoma; (d) positive cytoplasmic Bcl-2 immunostaining in a well differentiated serous ovarian carcinoma; (e) positive cytoplasmic Gadd45 immunostaining in a poorly differentiated adenocarcinoma of the ovary; (f) positive nuclear $p21^{WAF1/CIP1}$ immunostaining in a moderately differentiated endometrioid ovarian carcinoma (magnification×400); (g) positive nuclear p53 immunostaining in a poorly differentiated endometrioid adenocarcinoma; and (h) positive nuclear Mdm2 (Ab-1, Clone IF2 Oncogene Research Products) immunostaining in an ovarian carcinoma (magnification×200).

Table 2
Expression and association between p53-related proteins and established prognostic factors[a]

	Apoptosis $n=64$	Bax $n=66$	Bcl-2 $n=66$	Gadd45 $n=66$	Ki-67 $n=66$	Mdm2 $n=66$	$p21^{WAF1/CIP1}$ $n=66$	p53 $n=66$	Age	Residual disease	FIGO stage	Pathological subtype	Tumour grade
Apoptosis positive = 44 (69%)													
Bax strong = 29 (44%)													
Bcl-2 positive = 30 (45%)		++											
Gadd45 positive = 48 (73%)													
Ki-67 positive = 42 (64%)													
Mdm2 (Oncogene Res.) positive = 6 (9%)		+											
$p21^{WAF1/CIP1}$ positive = 51 (77%)				++									
p53 positive = 38 (58%)						+	+						
Age							+						
Residual disease													
FIGO stage													
Pathological subtype													
Tumour grade						+							
Performance status													

FIGO, International Federation of Gynaecologists and Obstetricians.

[a] +, $P<0.05$; ++, $P<0.01$.

if any of the malignant tumour cells were stained. There was no staining of any of the tissue in sections that were described as negative for Mdm2.

3.3. Results of immunostaining prechemotherapy

Sixty-four sections were immunostained for apoptosis, and 66 sections for the other biological factors, in prechemotherapy patients (Table 2). Thirty-eight (58%) of the tumour sections stained positively for p53 protein. Mdm2 expression was low and dependent on the antibody used. Only one tumour was positive using Mdm2 (DAKO); using Mdm2 (Oncogene Research Products) six sections were positive (including the section that had previously stained positive). Strong (nuclear) Bax expression was detected in 29 (44%) of sections and positive Bcl-2 expression in 30 (45%) of sections. Nuclear $p21^{WAF1/CIP1}$ was detected in 51 (77%) of the tumours while 48 (73%) showed Gadd45 expression. Forty-four (69%) of the sections showed positive immunofluorescence for apoptosis in the cancer cells. Nuclear Ki-67 staining, denoting malignant cell proliferation, was seen in 42 (64%) of the tumours.

3.4. Relationship between biological and established prognostic factors

The association between the expression of each of the different proteins and apoptosis prior to chemotherapy was examined using the χ^2 or Fisher's Exact Test (Table 2). There were significant associations between the expression of p53 and $p21^{WAF1/CIP1}$ ($P=0.04$), and between p53 and Mdm2 (Oncogene Research Products) ($P=0.035$). All 6 Mdm2-positive sections were p53 positive. The association between positive expression of p53 protein and strong (nuclear) expression of Bax also approached significance ($P=0.068$).

There was a strong association between Bcl-2-positive sections and strong (nuclear) Bax expression ($P=0.005$), but no association was observed between Bcl-2-positive sections and the other related proteins or apoptosis. Examining the association between Bax expression and the remaining factors, there was a significant association between strong Bax expression and Mdm2-positive tumours ($P=0.017$). Associations approaching significance were observed between strong Bax expression and Gadd45-positive sections ($P=0.082$), and between strong Bax expression and $p21^{WAF1/CIP1}$-positive sections ($P=0.065$). However, Bax expression was not associated with apoptosis or Ki-67 positive sections.

Tumour sections that were $p21^{WAF1/CIP1}$-positive were strongly associated with positive Gadd45 expression ($P=0.003$), but not with apoptosis, or expression of Bcl-2, Ki-67 or Mdm2. Ki-67-positive sections were not associated with any of the other biological factors.

The association between each of the standard prognostic factors and the biological factors was similarly analysed and age below the median of 55.5 years was associated with $p21^{WAF1/CIP1}$-positive sections ($P=0.04$); similarly age greater than the median approached significance in relationship to sections containing malignant apoptotic cells ($P=0.06$). There was an association between Mdm2-positive tumours and low-grade (well-differentiated) tumours ($P=0.022$). There was a relationship between Gadd45 expression and pathological subtype (negative Gadd45 expression with adenocarcinoma NOS or mixed tumours; positive Gadd45 expression with other subtypes) but this was not significant ($P=0.07$). It is important to note that a

Table 3
Univariate and multivariate analysis of biological and prognostic factors[a]

	Univariate analysis			Multivariate analysis		
	Response	Progression	Survival	Response	Progression	Survival
Apoptosis						
Bax	++			++		
Bcl-2						
Gadd45			+			
Ki-67		++	++		+	
Mdm-2						
$p21^{WAF1/CIP1}$						
p53	++			++		
Age at diagnosis						
Bulk residual disease	++	+				
FIGO Stage	++	++	++	++	++	++
Pathological subtype		+	+			
Tumour grade						
KPS						

KPS, Karnofsky Performance Status; FIGO, International Federation of Gynaecologists and obstetricians.
[a] +, $P<0.05$; ++, $P<0.01$.

large number of associations were examined and this may have led to the identification of some associations by chance.

3.5. Univariate and multivariate analysis — response to chemotherapy

A univariate analysis was performed for response to chemotherapy and each of the biological and standard prognostic factors (Table 3). 3 patients (FIGO stage Ic) were not included in the analysis, as the response to chemotherapy could not be assessed. In the remaining 63 patients, bulk residual disease ($P=0.0086$) and FIGO stage III or IV disease ($P=0.0044$) were associated with a partial or complete response to chemotherapy. Only two of six stage II tumours showed a response to chemotherapy, compared with 34 of 39 stage III tumours and 10 of 18 stage IV tumours. Both positive p53 expression ($P=0.00005$) and strong (nuclear) Bax expression ($P=0.0004$) were associated with partial or complete response in the univariate analysis.

In multivariate analysis, volume of residual disease no longer retained statistical significance. However, positive p53 expression ($P=0.0003$), strong Bax expression ($P=0.001$) and advanced FIGO stage ($P=0.009$) retained independent significance in association with response to chemotherapy (Table 3).

3.6. Univariate and multivariate analysis — disease progression

A univariate analysis was performed for PFS (Table 3). FIGO stage (I/II versus III versus IV) was significantly linked to PFS ($P<0.00005$), with a shorter time to progression as the stage advanced. The 5-year PFS for patients diagnosed with FIGO stage I/II disease was 70%; for stage III 16%; for stage IV 0%. Bulk residual disease ($P=0.03$) and pathological subtype ($P=0.04$) were also associated with a reduction in the PFS. The 5-year PFS for nil or minimal residual disease was 37% and for bulk residual disease 13%. The 5-year PFS for endometrioid tumours was 36%; serous tumours 26%; clear cell tumours 20%; malignant Brenner tumours 0%; mixed and adenocarcinoma NOS tumours 0%. There was no significant association between the PFS and age of the patient at initial diagnosis, WHO tumour grade (I versus II versus III) and KPS (90% versus 80% versus 30–70%).

Among the p53-related biological factors, negative Ki-67 expression was associated with an increase in the PFS ($P=0.00014$). The 5-year PFS for Ki-67-negative tumours was 44% and for Ki-67-positive tumours 0. Expression of the other biological factors did not approach statistical significance in relation to the PFS.

In multivariate analysis, both advanced FIGO stage and positive Ki-67 expression were independent factors associated with a reduction in PFS. The FIGO stage was the most important factor influencing the PFS ($P=0.00045$). However, expression of Ki-67 was still of significance ($P=0.024$). Neither residual disease, nor pathological subtype, retained statistical significance in multivariate analysis (Table 3).

3.7. Univariate and multivariate analysis — overall survival

Univariate analysis (Table 3) showed that the FIGO stage of the ovarian cancer was associated with survival ($P=0.0025$). FIGO stage was the most important of the standard prognostic factors on univariate analyses in relation to overall survival (Fig. 2). Pathological subtype was also associated with overall survival ($P=0.04$) (5-year survival for endometrioid tumours 75%; clear cell tumours 38%; serous tumours 35%; malignant Brenner tumours 8%; mixed or adenocarcinoma NOS tumours 0%). Among the individual biological factors,

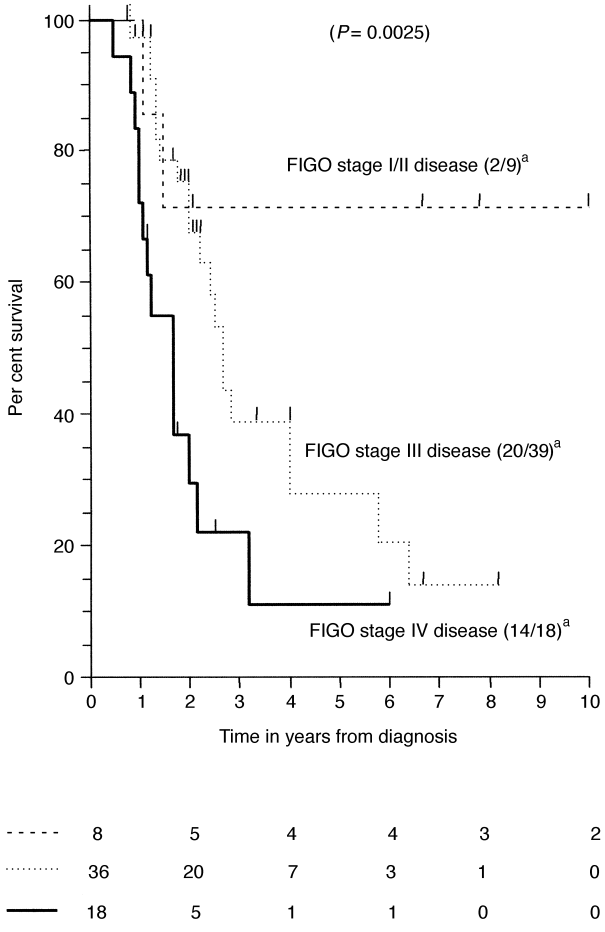

Fig. 2. Kaplan–Meier curve for International Federation of Gynaecologists and Obstetricians (FIGO) stage versus overall survival (univariate analysis). [a]Numbers in parentheses are the number of patients that have died in each subgroup.

negative Ki-67 expression ($P=0.003$) (Fig. 3) and negative Gadd45 expression ($P=0.03$) were associated with improved survival. The 5-year survival for patients with Ki-67-positive tumours was 11%, whereas for those with Ki-67-negative tumours it was 56%. The 5-year survival for patients with Gadd45-positive tumours was 18%; for those with Gadd45-negative tumours it was 60%.

Age of the patient, WHO tumour grade and KPS were not associated with overall survival. The 5-year survival was greater for patients with no residual or minimal residual disease (51%) versus bulk residual disease (24%), but this did not reach statistical significance ($P=0.08$).

Multivariate analysis (Table 3) showed that the FIGO stage was the most important factor influencing overall survival ($P=0.003$). However, there was still a trend between Ki-67-positive tumours and a reduction in overall survival that approached statistical significance ($P=0.055$). Pathological subtype and Gadd45 expression were no longer statistically significant.

3.8. Ratios of Bax/Bcl-2 and $p21^{WAF1/CIP1}$/Gadd45

In view of the significant association between positive Bcl-2 and strong (nuclear) Bax expression ($P=0.005$), the ratios of Bax and Bcl-2 were analysed for the association with response to chemotherapy, PFS and overall survival. However, there was no statistically significant association between Bax weak/Bcl-2-negative tumours, Bax strong/Bcl-2-negative tumours, Bax weak/Bcl-2-positive tumours, Bax strong/Bcl-2-positive tumours and response, progression or survival on univariate and multivariate analyses.

Similarly, in view of the significant association between positive $p21^{WAF1/CIP1}$ and positive Gadd45 expression ($P=0.003$), the ratios of $p21^{WAF1/CIP1}$ and Gadd45 were also analysed for the association with response, progression and survival. There was no association with response and overall survival. However, there was a significant association between the $p21^{WAF1/CIP1}$/Gadd45 ratio and response to chemotherapy ($P=0.01$), although this was based on small numbers of cases. There were 8 cases that were $p21^{WAF1/CIP1}$-negative/Gadd45-negative, with 6 recurrences and a 5-year PFS of 31%. There were 4 recurrences from four tumours that were $p21^{WAF1/CIP1}$-negative/Gadd45-positive, with a 5-year PFS of 0%. 6 out of 9 cases that were $p21^{WAF1/CIP1}$-positive/Gadd45-negative developed recurrent disease with a 5-year PFS of 27%. 30 of 39 cases that were $p21^{WAF1/CIP1}$-positive/Gadd45-positive developed recurrent disease with a 5-year PFS of 15%.

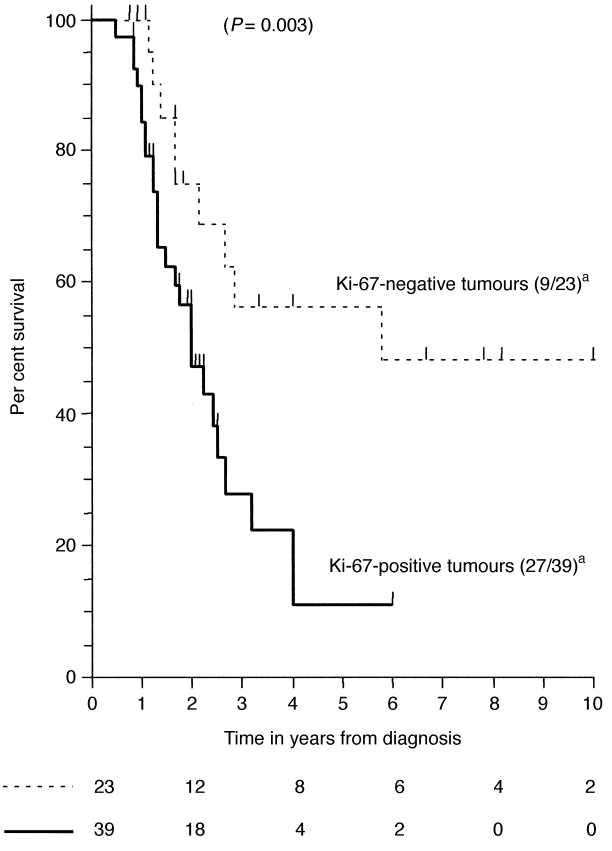

Fig. 3. Kaplan–Meier curve for Ki-67 expression versus overall survival (univariate analysis). [a]Numbers in parentheses are the number of patients that have died in each subgroup.

3.9. Relationship between biological factors in postchemotherapy samples

Comparison of 21 samples taken before and after chemotherapy did not reveal any statistically significant changes in any of the parameters (Table 4). p53 expression was unchanged in 17 samples (81%) after chemotherapy. Of four samples where expression differed, three samples changed from positive to negative and one from negative to positive. Similarly, Bcl-2 expression was unchanged in 14 (67%) of the paired tumours. Of the remaining samples, four changed from negative to positive and three from positive to negative. The paired samples all stained positively for Bax with 12 samples (57%) unchanged. Nine matched samples changed expression of Bax, with six changing from strong (nuclear) to weak (cytoplasmic) staining and three from weak to strong staining. Only one (5%) of the matched samples did not express $p21^{WAF1/CIP1}$, changing from positive to negative staining. All 21 of the matched samples (100%) expressed Gadd45 before and after chemotherapy. Ki-67 expression was unchanged in 13 (62%) matched samples. Of the remainder, five changed from positive to negative; three changed from negative to positive. The expression of Mdm2 in the paired samples was dependent on the antibody used. Using anti-Mdm2 antibody (DAKO), 18 (86%) samples remained negative after chemotherapy. However, three samples (14%) changed expression, from negative to

Table 4
Comparison of expression of biological factors in 21 matched pre- and postchemotherapy samples

Biological factor ($n=21$)	Negative staining prechemotherapy (%)	Negative staining postchemotherapy (%)	Positive staining prechemotherapy (%)	Positive staining postchemotherapy (%)	No. samples with changed expression (%)
Apoptosis	1 (5)	2 (10)	20 (95)	19 (90)	3 (14)
Bax	6[a] (29)	9[a] (43)	15[b] (71)	12[b] (57)	9 (43)
Bcl-2	10 (48)	9 (43)	11 (52)	12 (57)	7 (33)
Gadd45	0	0	21 (100)	21 (100)	0
Ki-67	6 (29)	8 (38)	15 (71)	13 (62)	8 (38)
Mdm2 (Clone SMP14)	21 (100)	18 (86)	0	3 (14)	3 (14)
Mdm2 (Ab-1, Clone IF2)	16 (76)	17 (81)	5 (24)	4 (19)	3 (14)
$p21^{WAF1/CIP1}$	0	1 (5)	21 (100)	20 (95)	1 (5)
p53	2 (10)	4 (19)	19 (90)	17 (81)	4 (19)

[a] Weak.
[b] Strong.

positive. For the anti-Mdm2 antibody (Ab-1) (Oncogene Research Products) 15 samples remained negative (71%) after chemotherapy, with three remaining positive after chemotherapy. Three samples changed expression, with two becoming negative after chemotherapy and one changing from negative to positive after chemotherapy.

4. Discussion

This is the first study to examine the independent significance of a broad range of p53-related proteins in comparison with established prognostic factors for epithelial ovarian cancer. The principal finding was that the FIGO stage was the most significant prognostic factor for response to chemotherapy, progression-free interval and overall survival. Ki-67 status was also an independent predictor of progression, and approached significance for survival on multivariate analysis, but interestingly not for response.

Bax and p53 expression were independently associated with response to chemotherapy. Recent data have shown that Bax expression is associated with an improved response to chemotherapy in ovarian cancer [32], which is consistent with its proposed role in the induction of apoptosis. In this study, Bax and Bcl-2 expression were associated and recent data have suggested that the ratio of Bax to Bcl-2 expression is the determinant of response [31,61,62] although we did not confirm this hypothesis in the present study.

The association between immunohistochemically detected p53 and response has not been reported in ovarian cancer [19,20]. When p53 is detected using immunohistochemistry, this is usually an indication of mutated [9,10] or dysregulated p53 [63], suggesting an abnormality in the apoptotic cascade. Thus, immunohistochemically-detectable p53 should predict for chemoresistance [19,20], yet there have been other reports that p53 immunopositive laryngeal cancer [64], bladder cancer [65] and head and neck cancer [66] respond better to chemotherapy. One hypothesis to explain the data would be that the cancer cells that express p53 do not undergo apoptosis while the rest of the cancer cells do die. However, we did not observe an increase in p53 expression in the 21 matched cases for which pre- and postchemotherapy specimens were available and recent data [67] have shown that spontaneous apoptosis is increased in p53 immunopositive ovarian cancers.

An alternative explanation for the paradoxical association between p53 expression and response might be related to the Mdm-2 and $p21^{WAF1/CIP1}$ proteins, which were significantly associated with p53 expression. While Mdm-2 suppresses p53 function, p21 is involved in the G1/S checkpoint cell cycle arrest. Thus, the apoptotic function of p53 might be suppressed in p53 immunopositive tumours where the protein is abnormal and dysfunctional. Here Mdm-2 may suppress the function of p53 or allow $p21^{WAF1/CIP1}$ to divert the cell into arrest rather than apoptosis. However, our study did not include co-localisation so that, although tumours contained p53 associated with Mdm-2 and $p21^{WAF1/CIP1}$, we do not have evidence that the proteins were co-expressed. In addition, we tested for a large number of associations in this study and, therefore, it is possible that some results achieved the level of statistical significance by chance.

The study has found that the FIGO stage is the most important predictor of the natural history of ovarian cancer and that Ki-67 status provides further information. The latter is of potential clinical significance as it could be used to define patients who might require a more aggressive clinical approach. A potential area of study is in patients with FIGO stage Ia or Ib disease who would normally undergo optimum surgical cytoreduction only. Prospective evaluation of Ki-67 in this population might identify patients who would benefit from adjuvant chemotherapy.

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References

1. Cancer Research Campaign. Ovarian Cancer — UK. Factsheet 17, 1997.
2. Parker SL, Tong T, Bolden S, *et al*. Cancer statistics. *CA Cancer J Clin* 1997, **47**, 5–27.
3. McGuire PM, Hoskins WJ, Brady MF, *et al*. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and stage IV ovarian cancer. *N Engl J Med* 1996, **334**, 1–6.
4. Levine AJ, Perry ME, Chang A, *et al*. The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumourigenesis. *Br J Cancer* 1994, **69**, 409–416.
5. Harris CC. The 1995 Walter Hubert Lecture — molecular epidemiology of human cancer: insights from the mutational analysis of the p53 tumour-suppressor gene. *Br J Cancer* 1996, **73**, 261–269.
6. Harris CC. p53 Tumour suppressor gene: from the basic research laboratory to the clinic — an abridged historical perspective. *Carcinogenesis* 1996b, **17**, 1187–1198.
7. Varley JM, Evans DGR, Birch JM. Li-Fraumeni syndrome — a molecular and clinical review. *Br J Cancer* 1997, **76**, 1–14.
8. Iggo R, Gatter K, Bartek J, *et al*. Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 1990, **335**, 675–679.
9. Skilling JS, Sood A, Niemann T, *et al*. An abundance of p53 null mutations in ovarian carcinoma. *Oncogene* 1996, **13**, 117–123.
10. Greenblatt MS, Bennett WP, Hollstein M, *et al*. Mutations in the p53 tumour suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994, **54**, 4855–4878.
11. Henriksen R, Strang P, Wilnder E, *et al*. p53 Expression in epithelial ovarian neoplasms: relationship to clinical and pathological parameters, Ki-67 expression and flow cytometry. *Gynaecol Oncol* 1994, **53**, 301–306.
12. Levesque MA, Katsaros D, Zola P, *et al*. Mutant p53 protein overexpression is associated with poor outcome in patients with well or moderately differentiated ovarian carcinoma. *Cancer* 1995, **75**, 1327–1338.
13. Klemi P, Pylkkänen L, Kiilholma P, *et al*. p53 Protein detected by immunohistochemistry as a prognostic factor in patients with epithelial ovarian carcinoma. *Cancer* 1995, **76**, 1201–1208.
14. Diebold J, Baretton G, Felchner M, *et al*. bcl-2 Expression, p53 accumulation and apoptosis in ovarian carcinomas. *Am J Clin Pathol* 1996, **105**, 341–349.
15. Herod JJO, Eliopoulos AG, Warwick J, *et al*. The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. *Cancer Res* 1996, **56**, 2178–2184.
16. Geisler JP, Geisler HE, Wiemann MC, *et al*. Quantification of p53 in epithelial ovarian cancer. *Gynaecol Oncol* 1997, **66**, 435–438.
17. Viale G, Maisonnneuve P, Bonoldi E, *et al*. The combined evaluation of p53 accumulation and of Ki-67 (MIB1) labeling index provided independent information on overall survival of ovarian carcinoma patients. *Annals Oncol* 1997, **8**, 469–476.
18. Röhlke P, Milde-Langosch K, Weyland C, *et al*. p53 Is a persistant and predictive marker in advanced ovarian carcinomas: multivariate analysis including comparison with Ki-67 immunoreactivity. *J Cancer Res Clin Oncol* 1997, **123**, 496–501.
19. Righetti SC, Della Torre G, Pilotti S, *et al*. A comparative study of p53 gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. *Cancer Res* 1996, **56**, 689–693.
20. Buttitta F, Marchetti A, Gadducci A, *et al*. p53 alterations are predictive of chemoresistance and aggressiveness in ovarian carcinomas: a molecular and immunohistochemical study. *Br J Cancer* 1997, **75**, 230–235.
21. Marks JR, Davidoff AM, Kerns BJ, *et al*. Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer Res* 1991, **51**, 2979–2984.
22. Niwa K, Itoh M, Murase T. Alteration of p53 gene in ovarian carcinoma: clinicopathological correlation and prognostic significance. *Br J Cancer* 1994, **70**, 1191–1197.
23. Hartmann LC, Podratz KC, Keeney GL, *et al*. Prognostic significance of p53 immunostaining in epithelial ovarian cancer. *J Clin Oncol* 1994, **12**, 64–69.
24. Eltabbakh GH, Belinson JL, Kennedy AW, *et al*. p53 overexpression is not an independent prognostic factor for patients with primary epithelial ovarian cancer. *Cancer* 1997, **80**, 892–898.
25. Dong Y, Walsh MD, McGukin MA, *et al*. Reduced expression of retinoblastoma gene product (pRB) and high expression of p53 are associated with poor prognosis in ovarian cancer. *Int J Cancer (Pred Oncol)*, 1997, **74**, 407–415.
26. Silvestrini R, Daidone MG, Veneroni S, *et al*. The clinical predictivity of biomarkers of stage III–IV epithelial ovarian cancer in a prospective randomized treatment protocol. *Cancer* 1998, **82**, 159–167.
27. Reed JC. Double identity for proteins of the Bcl-2 family. *Nature* 1997, **387**, 773–776.
28. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993, **74**, 606–619.
29. Miyashita T, Reed JC. Tumour suppressor p53 is a direct transcriptional activator of the human Bax gene. *Cell* 1995, **80**, 293–299.
30. Henriksen R, Wilander E, Oberg K. Expression and prognostic significance of Bcl-2 in ovarian tumours. *Br J Cancer* 1995, **72**, 1324–1329.
31. Marx D, Binder C, Meden H, *et al*. Differential expression of apoptosis associated genes Bax and Bcl-2 in ovarian cancer. *Anticancer Res* 1997, **17**, 2233–2240.
32. Tai Y, Lee S, Niloff E, *et al*. Bax protein expression and clinical outcome in epithelial ovarian cancer. *J Clin Oncol* 1998, **16**, 2583–2590.
33. El-Diery WS, Harper JW, O'Connor PM, *et al*. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* 1994, **54**, 1169–1174.
34. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993, **366**, 701–704.
35. Harper JW, Adami GR, Wei N, *et al*. The p21-cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependant kinases. *Cell* 1995, **75**, 805–816.
36. Waga S, Hannon GJ, Beach D, *et al*. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 1994, **369**, 574–578.
37. Flores-Rozas H, Kelman Z, Dean FB, *et al*. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc Natl Acad Sci USA* 1994, **91**, 8655–8659.
38. Barboule N, Mazars P, Baldin V, *et al*. Expression of p21WAF1/CIP1 is heterogeneous and unrelated to proliferation index in human ovarian carcinoma. *Int J Cancer* 1995, **63**, 611–615.

39. Werness BA, Jobe JS, DiCioccio RA, *et al*. Expression of the p53 induced tumour suppressor p21waf1/cip1 in ovarian carcinomas: correlation with p53 and Ki-67 immunohistochemistry. *Int J Gynaecol Pathol* 1997, **16**, 149–155.
40. Anttila MA, Kosma VM, Hongxiu J, *et al*. p21/WAF1 expression as related to p53, cell proliferation and prognosis in epithelial ovarian cancer. *Br J Cancer* 1999, **79**, 1870–1878.
41. Smith ML, Chen IT, Zhan Q, *et al*. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* 1994, **266**, 1376–1380.
42. Cahilly-Snyder L, Yang-Feng T, Francke U, *et al*. Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somatic Cell Mol Genet* 1987, **13**, 235–244.
43. Foulkes WD, Stamp GW, Afzal S, *et al*. MDM2 overexpression is rare in ovarian carcinoma irrespective of TP53 mutation status. *Br J Cancer* 1995, **72**, 883–888.
44. Muggia FM, Krezorski SK, Hansen H. Cell kinetic studies in patients with small cell carcinoma of the lung. *Cancer* 1974, **34**, 1683–1690.
45. Schwartz BR, Pinkus G, Bacus S, *et al*. Cell proliferation in non-Hodgkins lymphoma. Digital image analysis of Ki-67 antibody staining. *Am J Pathol* 1989, **134**, 327–336.
46. Hall PA, Levison DA. Review: assessment of cell proliferation in histological material. *J Clin Pathol* 1990, **43**, 184–192.
47. Barnabei VM, Miller DS, Bauer KD, *et al*. Flow cytometric evaluation of epithelial ovarian cancer. *Am J Obstet Gynaecol* 1990, **162**, 1584–1590.
48. Isola J, Kallioniemi OP, Korte JM, *et al*. Steroid receptors and Ki-67 reactivity in ovarian cancer and in normal ovary: correlation with DNA flow cytometry, biochemical receptor assay, and patient survival. *J Pathol* 1990, **162**, 295–301.
49. Henriksen R, Strang P, Wilander E, *et al*. p53 expression in epithelial ovarian neoplasms: relationship to clinical and pathological parameters, Ki-67 expression and flow cytometry. *Gynaecol Oncol* 1994, **53**, 301–306.
50. Kerns BJ, Jordan PA, Faerman LL, *et al*. Determination of proliferation index with MIB-1 in advanced ovarian cancer using quantitative image analysis. *Am J Clin Pathol* 1994, **101**, 192–197.
51. Thomas H, Nasim MM, Sarraf CE, *et al*. Proliferating cell nuclear antigen (PCNA) immunostaining — a prognostic factor in ovarian cancer? *Br J Cancer* 1995, **71**, 357–362.
52. Viale G, Maisonneuve P, Bonoldi E, *et al*. The combined evaluation of p53 accumulation and of Ki-67 (MIB1) labelling index provides independent information on overall survival of ovarian carcinoma patients. *Ann Oncol* 1997, **8**, 469–476.
53. Garzetti GG, Ciavattini A, Goteri G, *et al*. Ki67 antigen immunostaining (MIB 1 monoclonal antibody) in serous ovarian tumours: index of proliferative activity with prognostic significance. *Gynaecol Oncol* 1995, **56**, 169–174.
54. Hartmann LC, Sebo TJ, Kamel NA, *et al*. Proliferating cell nuclear antigen in epithelial ovarian cancer: relation to results at second-look laparotomy and survival. *Gynaecol Oncol* 1992, **47**, 191–195.
55. Anttila M, Kosma VM, Ji H, *et al*. Clinical significance of alpha-catenin, collagen IV, and Ki-67 expression in epithelial ovarian cancer. *J Clin Oncol* 1998, **16**, 2591–2600.
56. Yamasaki F, Tokunaga O, Sugimori H. Apoptotic index in ovarian carcinoma: correlation with clinicopathologic factors and prognosis. *Gynaecol Oncol* 1997, **66**, 439–448.
57. Shepherd JH. Revised FIGO staging for gynaecological cancer. *Br J Obstet Gynaecol* 1989, **96**, 889–892.
58. Fox H, Buckley CH. Neoplasms of the ovary. In *Pathology for Gynaecologists*, 2nd ed. London, Hodder and Stoughton, 1991.
59. Serov SF, Scully RE, Sobin LH. *International Histologic Classification and Staging of Tumours. No. 9 Histologic Typing of Ovarian Tumours*. Geneva, Switzerland, World Health Organisation, 1973.
60. McKee PH, Hobbs C, Hall PA. Antigen retrieval by microwave irradiation lowers immunohistological detection thresholds. *Histopathology* 1993, **23**, 377–379.
61. Chresta CM, Masters JR, Hickman JA. Hypersensitivity of human testicular tumours to etoposide-induced apoptosis is associated with functional p53 and a high Bax:Bcl-2 ratio. *Cancer Res* 1996, **56**, 1834–1841.
62. Eliopoulos AG, Kewrr DJ, Herod J, *et al*. The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene* 1995, **11**, 1217–1228.
63. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. *Nature* 1991, **351**, 453–456.
64. Bradford CR, Zhu S, Wolf GT, *et al*. Overexpression of p53 predicts organ preservation using induction chemotherapy and radiation in patients with advanced laryngeal cancer. Department of Veterans Affairs Laryngeal Cancer Study Group. *Otolaryngol Head Neck Surg* 1995, **113**, 408–412.
65. Sengelov L, Horn T, Steven K. p53 nuclear immunoreactivity as a predictor of response and outcome following chemotherapy for metastatic bladder cancer. *J Cancer Res Clin Oncol* 1997, **123**, 565–570.
66. Giatromanolaki A, Koukourakis M, Zaramboukas T, *et al*. p53 and bcl-2 expression in locally advanced squamous cell head-neck cancer treated with platinum based chemotherapy and radiotherapy. *Anticancer Res* 1998, **18**, 4685–4692.
67. Kupryjanczyk J, Dansonka-Mieszkowska A, Szymanska T, *et al*. Spontaneous apoptosis in ovarian carcinomas: a positive association with p53 gene mutation is dependent on growth fraction. *Br J Cancer* 2000, **82**, 579–583.