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P53 Mutation in a Series of Epithelial Ovarian Cancers from the U.K., and its Prognostic Significance

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In an initial study of 20 fresh ovarian tumour samples, we compared the immunohistochemical positivity of staining of the p53 protein with the presence of missense mutations of the *P53* gene. This revealed a prevalence of 50% with a perfect correlation between mutation and immunohistochemical staining. Detection of the p53 protein by immunohistochemistry was, therefore, used as a reliable indicator for the presence of *P53* mutation, and was applied to a study of an archival series of 93 ovarian tumours. Positive immunostaining of the p53 protein was observed in 47% of this series. Cox regression was used to assess whether various clinical variables and *P53* mutation were related to survival. As a result, it was found that positive staining of the p53 protein was independent of age, tumour differentiation, tumour type, though possibly not stage. There was some evidence that p53 positivity was associated with reduced survival after adjusting for other variables, but the result was not statistically significant.

Keywords: *P53*, mutation, ovarian cancer, survival
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INTRODUCTION

OVARIAN CANCER is one of the most common causes of cancer death in females in the U.K., with an estimated 4400 new cases and 3800 deaths from the disease each year [1]. The majority of these cases occur in the over-45 age group, and usually present late, because symptoms are initially vague so that the majority of patients have stage 3 or 4 disease at diagnosis [2]. Due to its relatively high frequency, and to the complexities of the treatment regimens, the management of ovarian cancer was the subject of the First Report of the Standing Sub-committee on Cancer by the UK Department of Health. One of the greatest potential benefits suggested by this report was the reduction in the morbidity caused by inappropriate treatment [3]. Even to achieve this limited goal, however, it would be essential to identify prognostic factors of greater reliability than those currently available.

The *P53* gene is currently regarded as the most important gene in the evolution of human malignant disease. Not only is it associated with a wide spectrum of neoplasms, but it is also found to be frequently mutated or deleted within any one tumour type [4, 5]. Its protein product is a nuclear phosphoprotein [6, 7] which appears to be constitutively expressed at low levels in all mammalian cells. By contrast the p53 protein in transformed cells appears to be present at elevated levels, due primarily to its longer half-life in these cells [8, 9]. The most common mechanism which results in the presence of a stable form of p53 protein is the occurrence of a missense mutation. Mutations of *P53* are common events, especially in aggressive tumours, such as lung cancer [10], and it is possible that they may be useful as markers of clinical outcome [11].

A number of studies of ovarian cancer have demonstrated allelic loss of chromosome 17 [12], positivity of p53 immunohistochemical staining [13, 14] and mutation of the *P53* gene [15–17]. However, of two studies which have attempted to relate such *P53* mutations to patient survival, one did not explicitly examine survival [18] and the second did not adjust for other prognostic variables [19]. Using the chemical mismatch cleavage technique [20], we have recently been able to identify missense mutations of the *P53* gene in 50% of a series of fresh ovarian tumours [21]. Although it has been suggested that such mutations may not invariably agree with positive immunohistochemical staining of the p53 protein [22], we present evidence here that, in ovarian cancer, the positivity of staining is a reliable indicator of *P53* mutation. This has, therefore, allowed us to use immunohistochemistry to assess the presence of *P53* mutations in a series of archival tumour samples, and hence to evaluate the relationship of *P53* mutation to patient survival.

PATIENTS AND METHODS

Patients and tissue

94 patients underwent surgery in the same unit between 1985–1991, and almost all received adjuvant chemotherapy with single-agent platinum regimens of the kind recommended in the U.K. The patients were followed up for at least 2 years.

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Survival was recorded in whole months, up to the follow-up date (November 1992), at which point living patients were censored.

Duplicate 5-μm sections were cut from blocks of formalin-fixed, paraffin-embedded tissue. One section was processed for haematoxylin/eosin staining and the other analysed for p53 expression.

Immunohistochemical staining

Sections were dehydrated and deparaffinised by passing them through a graduated series of alcohols and xylenes. Endogenous tissue peroxidase activity was blocked by incubating the sections for 15 min in an 0.03% solution of hydrogen peroxide. This was rinsed off with sterile phosphate-buffered saline and the sections incubated consecutively with rabbit anti-mouse antibody to block non-specific binding and then, after further rinsing, with the D07 anti-p53 antibody (NovoCastra), in a humidified chamber for 60 min. Bound antibody was detected with a biotinylated rabbit anti-mouse antibody, and an avidin–biotin complex linked to horseradish peroxidase. The final product was visualised by using diaminobenzidine (1 mg/ml) in the presence of 0.03% hydrogen peroxide, and the sections lightly counterstained with Mayer's haematoxylin before clearing and mounting in an aqueous medium. Negative controls were performed by omitting the primary antibody, and positive control tissue (a colon carcinoma with a confirmed missense mutation) was included in every run.

Statistical analysis

Exact confidence limits for the proportion of agreement between positive immunochemical staining and the presence of missence mutations were found using Documenta Geigy tables. The association of the predictor variables with survival was assessed by Cox regression [23], while the association between p53 staining and categorical predictor variables such as stage, differentiation and type was assessed by χ^2 tests (using a trend test when the categories were ordered as in stage). The relationship between p53 staining and age was assessed by a two-sample *t*-test.

RESULTS

Initially, we studied 20 patients, who had recently presented with epithelial ovarian cancer, to assess the reliability of immunohistochemical staining of the p53 protein as an indicator for the presence of missense mutations in the *P53* gene. We had previously identified *P53* missense mutations in these samples by chemical mismatch cleavage and direct sequencing of polymerase chain reaction amplified fragments representing exons 2–9 of the gene [21]. Although these ovarian tumours were freshly obtained, we carried out the immunohistochemistry on paraffin sections of this material so that the resulting staining data could be reliably evaluated for its use in analysing an archival series of ovarian tumours.

There were two distinct immunohistochemical patterns of staining for p53 protein. Either there was no evidence of staining (Figure 1a) or, alternatively, the majority of the cells in the section exhibited strong positive staining of the p53 protein (Figure 1b). In some samples, the tumour cells were present only in focal areas of the section, and the immunopositivity reflected this pattern. The assignment of the samples to either positive or negative staining patterns was, therefore, straightforward. All immunostaining was confined to the nuclei of the cells concerned (Figure 1c), and we observed no cytoplasmic staining of the type reported in breast [24] or lung cancers [10]. Staining within the tumour cells was granular or reticular in nature, and

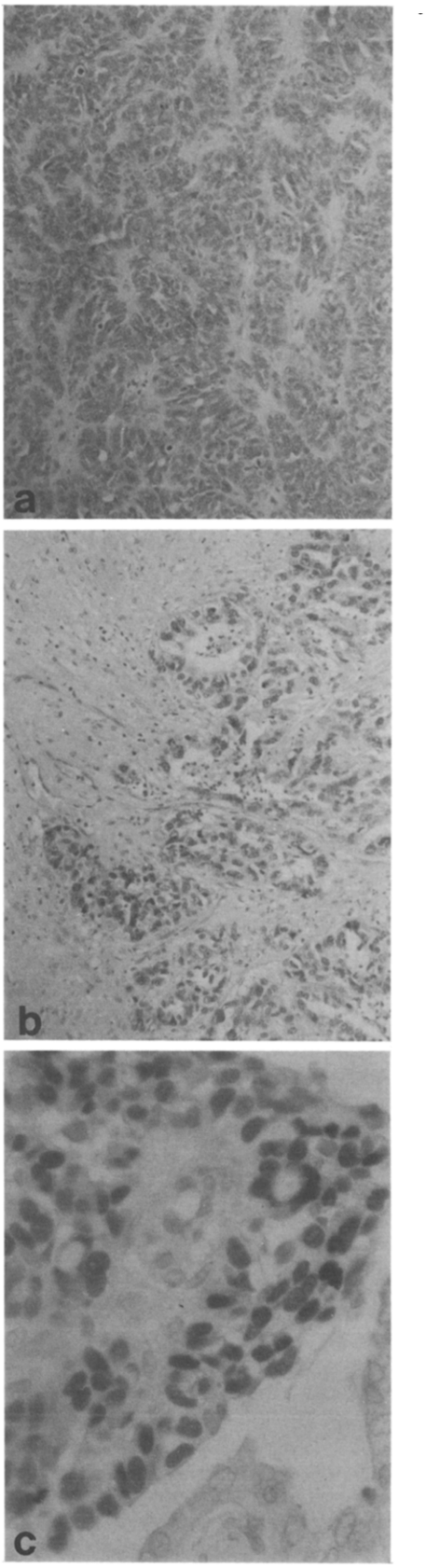

Figure 1. Immunohistochemical staining of the p53 protein in paraffin sections of human ovarian tumours. (a) Negative staining of tumour cells, (b) positive staining of tumour cells, (c) higher magnification of positive-stained cells demonstrating nuclear localisation of p53 protein.

during mitosis the reaction product was dramatically reduced (data not shown) in a manner similar to that reported in colon tumours [25].

The data for the 20 ovarian tumour samples (patients 01–20) are summarised in Table 1. Comparison of these results with the data from our previous mutation study [21], revealed perfect agreement between the presence of a missense mutation in the *P53* gene and positive staining of the p53 protein. Although this does not prove that the two parameters are always correlated in ovarian tumours, as the lower 95% confidence limit would be 86% (kappa = 1.00, 95% confidence limits 0.72–1.00), there is clearly a close relationship between them.

We investigated a total of 93 ovarian tumour samples for evidence of positive immunohistochemical staining of the p53 protein. The results from this study are summarised in Table 2, and demonstrate that 44/93 (47%) of the samples exhibited positive staining for the p53 protein. This table also summarises the relationships between p53 staining and stage, differentiation, tumour type and age. Positive staining of p53 protein appeared to correlate with more advanced tumour stage, but not with age, tumour type or differentiation.

Stage, age (given stage) and differentiation (given age and stage), showed highly significant associations with survival. Tumour type (given the previous variables) was marginally significant. It could be argued that these variables might have been fitted *a priori* to the model without formal testing. However, it was felt that their effects ought to be tested in order to conserve degrees of freedom should any not prove significant. The effect of p53 status given age, stage and differentiation was then assessed, but was not statistically significant (Table 3); the relative risk of dying if p53 immunopositive was greater than one (1.44) with wide confidence limits (95% confidence limits 0.73–2.83).

DISCUSSION

We have analysed a series of patients with ovarian cancer for evidence of the presence of mutant p53 protein in their tumour cells, and have attempted to correlate these data with a variety of clinical parameters. The clinical details of the 93 patients included in this study indicated that the patients represented a typical U.K. series for this type of disease.

Immunohistochemical analysis of the p53 protein has been used as an indicator of mutation of the *P53* gene in a wide range of malignancies, but this relationship has recently been questioned [22, 26]. We have demonstrated in 20 of the ovarian tumours, however, that the D07 anti-p53 antibody produced positivity of staining in archival tissue correlated exactly with the presence of missense mutations in the *P53* gene.

It has previously been suggested that *P53* mutations are a late event involved in the progression of ovarian cancer [27], and, although in the present study p53-positive tumours were present in all anatomical stages of the disease, there was some evidence for a similar relationship. Conflicting conclusions have been reported from studies of gastric carcinomas [28] and breast tumours [29, 30].

Table 1. Comparison of mutation status of P53 *gene with immunohistochemical staining of the p53 protein*

Patient no.	Mutation of *P53* gene*	Staining of p53 protein
01	Missence mutation	+
02	Missence mutation	+
03	—	−
04	Missence mutation	+
05	Missence mutation	+
06	Missence mutation	+
07	—	−
08	—	−
09	—	−
10	—	−
11	—	−
12	—	−
13	Missence mutation	+
14	—	−
15	Missence mutation	+
16	Missence mutation	+
17	Missence mutation	+
18	Polymorphism	−
19	—	−
20	Missence mutation	+

* Data obtained from [21].

Table 2. Association between p53 staining and other predictor variables

Variable	p53+	p53−	Statistical analysis
Stage			
1	7	16	
2	7	12	
3	27	18	
4	3	3	Trend $\chi^2 = 4.902$ (df = 1), $P = 0.027$
Differentiation			
Well	6	6	Trend $\chi^2 = 2.068$ (df = 1),
Moderate	13	26	$P = 0.150$
Poor	25	17	
Tumour type			
Mucinous	8	16	Homogeneity $\chi^2 = 3.497$
Clear cell	6	3	(df = 3), $P = 0.321$
Serous	22	23	
Endometrial	8	7	
Mean age (years)	55.9	59.0	$t = 1.19$ (df = 1), $P = 0.238$

df, degrees of freedom.

Table 3. Significance of p53 positivity of staining and other clinical variables

Variables	χ^2 value	Degrees of freedom	2-tailed *P* value (to three decimal places)
Stage alone	45.20	3	0.000
Age given stage	5.72	1	0.017
Tumour differentiation given age and stage	6.35	2	0.042
Tumour type given differentiation and age and stage	2.38	3	0.497
p53 mutation given differentiation and age and stage	1.14	1	0.286

In this series, the presence of mutant p53 protein did not appear to be useful in indicating outcome in epithelial ovarian cancer, although, because of the wide confidence limits, this possibility cannot be completely ruled out from our data. This is in agreement with findings from a study of lung cancers [31], although the situation in tumours of the colon [32] and breast [28, 33] remains unclear. G:C transitions constitute the majority of colon tumour mutations (79%), the majority of which occur at CpG dinucleotides, while in sporadic breast tumours only a minority of mutations occur at these sites [4]. The unusual susceptibility of CpG sites is due to the presence of 5-methylcytosine residues and the ease with which they undergo spontaneous deamination to thymine. This event occurs slowly, but the rate of deamination can be increased by the presence of oxygen radicals, which are produced by the action of nitrosamines in the gut. This may, therefore, be the main mechanism for generating *P53* mutations in colon cancer. In lung cancers, where the incidence of mutations at CpG sites account for only 8% of abnormalities, and transversions are the most common form of mutation, it has been proposed that exposure to carcinogens in tobacco smoke account for the different pattern of mutation. It has been suggested that mutations at the CpG dinucleotides at codons 248, 173 and 289 are associated with a worse outcome [34]. The pattern of mutation we observed in ovarian cancers [21] is similar to that reported in sporadic breast cancer, with about 20% of transitions occurring at CpG sites. Until recently, it was thought that all *P53* mutations were functionally equivalent, but more recent data have indicated that different mutations may result in distinct phenotypes [34]. If this is the case in ovarian tumours, then the resulting pleiotropy may mean that it is not possible to interpret *P53* mutation as a prognostic factor when it is assessed simply by immunohistochemical staining of the p53 protein.

In conclusion, we have shown that in ovarian cancer immunohistochemical staining of the p53 protein in archival samples correlates with mutation of the *P53* gene. We did not find it to be a strong predictor of outcome in these patients, but we should not be surprised if it were found to be weakly predictive in another, larger, series of patients. It is also possible that correlations of survival with the exact type of mutation may still prove to be useful.

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