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Original Paper

Mutant p53 Protein as a Predictor of Survival in Endometrial Carcinoma

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The expression of mutated p53 protein was studied in paraffin-embedded, formalin-fixed tumour specimens from 183 women with endometrial carcinoma. Fifty-five per cent of the specimens were negative, whereas the staining intensity was weak, moderate or strong in 15, 2 and 28% of cases, respectively. Strong p53 expression (>75% of the cells stained) was more common in uterine papillary serous cancers and clear cell cancers than in other tumour subtypes ($P < 0.001$), as well as in poorly differentiated tumours ($P < 0.01$) and in tumours with nuclear grade 3 ($P < 0.0001$). Strong p53 expression was also more frequently found in aneuploid tumours ($P < 0.0001$) and in tumours with a high S-phase fraction ($P < 0.001$). Strong p53 expression was highly predictive of poor survival in the univariate analysis ($P = 0.006$) and in the Cox multivariate analysis which included age, stage and grade. However, it lost most of its impact when the strongly prognostic nuclear grade and ploidy were added to the multivariate models. Copyright © 1996 Published by Elsevier Science Ltd

Key words: endometrial carcinoma, prognosis, p53, DNA ploidy, proliferation, nuclear grade
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INTRODUCTION

ENDOMETRIAL carcinomas clinically confined to the uterus, i.e. stages I–II, are known to have a favourable prognosis and mortality is on the decrease [1]. In the clinical situation, therapy is generally based on stage, histopathological grade and the degree of myometrial invasion [2, 3], but these factors are not sensitive enough to identify well-defined high-risk and low-risk groups. Thus, the majority of deaths, in absolute numbers, still occurs within stage I–II patients, since 90% of the patients are confined to this group and only 10% of patients present with stage III–IV tumours. It would therefore be of value to find more objective prognostic factors before treatment is initiated.

One such potential prognostic factor is the mutated p53 tumour supressor gene product, which has proven to be of prognostic value in ovarian carcinoma [4, 5], gastrointestinal carcinomas [6] and in breast cancer [7]. The *TP53* supressor gene codes for a 53 kD nuclear phosphoprotein, which is expressed in most normal cells. The 'wild type' protein has important regulatory mechanisms in the cell cycle. If the DNA is damaged, the p53 product accumulates intracellularly through a post-translational stabilisation mechanism and arrests the cell cycle at G1, thus allowing extra time for repair. If repair fails, p53 may trigger deletion of cells by apoptosis [8]. 'Wild type' p53 cannot be detected with immunohistochemical methods, since its half-life is only 6–20 min [8].

Mutations of the *TP53* gene are common in malignancies. The mutated gene product loses its regulatory capacity and the half-life of this protein is prolonged to 4–8 h. Therefore, the mutated p53 protein can be detected in formalin-fixed, paraffin-embedded tissues with immunohistochemical methods. The aim of this study was to relate mutated p53 protein expression to clinical variables, DNA ploidy and S-phase fraction and to evalute its prognostic impact in univariate and multivariate models.

PATIENTS AND METHODS

Patients

This prospective study comprised 183 patients with invasive endometrial carcinoma of medium or high risk. The majority of patients with well differentiated clinical stage I adenocarcinomas and certain patients with moderately differentiated stage I carcinomas, i.e. low-risk patients, underwent primary sur-

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gery at their local hospitals and were not included, whereas women with a high risk of surgical complications (age, general condition) and patients who refused surgery were treated with radiotherapy and included in the study. Their mean age was 69 years (range 43–91 years) and 173 women (95%) were postmenopausal. No patient was lost to follow-up.

Staging

Prior to treatment, clinical staging according to FIGO [9] was performed. 72 patients (39%) were stage I, 91 (50%) stage II, 13 (7%) stage III and 7 (4%) stage IV. All underwent fractional curettage prior to treatment. Specimens were taken and divided for histopathological examination, and flow cytometry. The flow cytometric results were only used if the histopathological diagnosis of the corresponding tissue was conclusively malignant.

Histopathology

Histopathological grading was performed according to WHO criteria. In 51 cases the tumours (28%) were well differentiated, 76 (41%) were moderately differentiated, 53 (29%) poorly differentiated and 3 (2%) were undifferentiated. Nuclear grading was grouped into three categories [10].

Flow cytometry (FCM)

DNA analysis (material available in 159 cases) was performed using flow cytometry of fresh-frozen tissue. All the specimens were preserved in 12% DMSO solution and instantly frozen at −70°C. Before preparation, the specimens were thawed at room temperature for 60–90 min. Single cell nuclei were prepared according to a method previously described [11], with certain modifications after 1992 [12, 13]. The DNA values were expressed in relative DNA values (ploidy level), where the diploid content of normal cells was given the value of 2.0c. Human lymphocytes with a diploid DNA content (2.0c) and with a coefficient of variation (CV) of 1–2% were used as an external standard. Tumours between 1.8c and 2.2c (2.0c included) were regarded as peridiploid tumours, whereas those diverging from the values mentioned were regarded as grossly aneuploid.

Immunohistochemistry: p53

Five micrometre sections of paraffin-embedded, formalin-fixed tumour tissues were used for the study. To enhance the staining, each slide was subjected to microwave antigen retrieval (MWAR) (900 W, 3 × 5 min). The slides were stained with a monoclonal antibody, p53/DO-7 (Novocastra UK, Bio-Zac), according to a standardised avidin–biotin method, provided by the manufacturers. The sections were then counterstained in Meyer's haematoxylin. All slides were reviewed independently by two of the authors. The extent of p53 immunoreaction was scored visually according to the following: negative staining = no detectable staining, weak ≤15% of cells stained, moderate >15%–≤75% and strong >75% of cells stained.

Statistical analysis

The Cox proportional hazards model was used in the univariate and multivariate analyses. The model is explained in detail elsewhere [14]. Chi-square tests were used when proportions were compared.

RESULTS

Descriptive p53 immunohistochemistry studies

In 101 cases the tumours (55%) had no detectable staining of p53, whereas weak, moderate or strong staining was found in 27 (15%), four (2%) and 51 (28%) tumours, respectively. Strong p53 expression was more frequent in stages III and IV (38 and 57%) than in stages I and II (26 and 24%), although the difference was not significant. As regards histopathological subtypes, cases of UPSC-type (uterine papillary serous carcinoma, $n = 20$) or clear cell carcinoma ($n = 2$) had strong p53 expression more often than the other tumour types ($P < 0.001$). The proportion also increased with histopathological dedifferentiation and was found in 14–25–41–67% of the well–moderately–poorly and undifferentiated tumours ($P < 0.01$).

Strong p53 expression was also associated with gross nuclear aberrations and proliferation and was, therefore, significantly more common in tumour specimens with nuclear grade 3 (46%) than in grade 1 or 2 tumours (13%) ($P < 0.0001$). Ninety-five tumours (60%) were peridiploid (range 1.8c–2.2c, of which 69 had the value of 2.0c and 87 out of 95 had values of 1.9c–2.0c–2.1c), whereas 64 (40%) were grossly aneuploid. Strong p53 expression was strongly associated with aneuploidy ($P < 0.0001$) and high S-phase fractions ($P < 0.001$): the proportions in peridiploid and aneuploid tumours were 11 and 58%, respectively, and 18 and 54% in tumours with S-phase fractions < or ≥15%.

Prognosis

Univariate analysis. Strong expression of the mutated p53 protein (>75% tumour cells stained) was a powerful, significant predictor of poor outcome, and relative hazard (RH) was 2.29 (95% confidence limit 1.44–3.67) ($P = 0.0006$) (Figure 1). The RH for weakly and moderately stained tumours was 0.47, but the confidence interval (0.20–1.11) included RH = 1, so their prognostic impact was not significantly different from that of the reference group, i.e. the negative tumours.

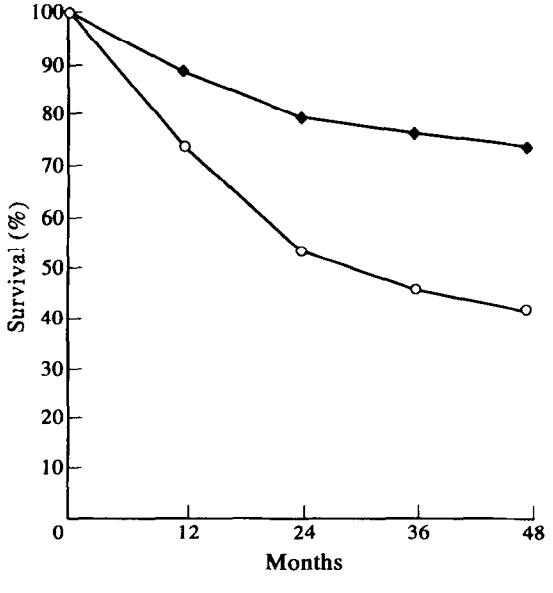

Figure 1. Cancer-specific survival for patients with strong p53 stainings (p53 > 75% of cells stained, ○) and others (p53 ≤ 75% of cells stained ◆).

Table 1. Multivariate analysis of clinical, histopathological variables and p53 in relation to disease-specific survival (n = 180, *events* = 74)

Variable	Categories	Relative hazard	95% confidence limits	*P* value
Age	Continuous	1.049	1.02–1.07	***
Clinical stage	I	1.0	Ref.	
	II	1.08	0.64–1.84	NS
	III	3.78	1.69–8.44	***
	IV	7.46	2.91–19.12	***
Differentiation	Well	1.0	Ref.	
	Moderate	1.22	0.65–2.29	NS
	Poor	1.64	0.81–3.33	NS
p53	No staining	1.0	Ref.	
	Weak or moderate staining	0.63	0.26–1.56	NS
	Strong staining	1.99	1.17–3.38	**

NS, non-significant; Ref, reference group; *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Strong staining > 75% of cells stained. Weak and moderate staining ≤ 75% of cells stained.

Nuclear grade 3 and aneuploidy were also strongly associated with a poor outcome ($P < 0.01$ and $P < 0.001$, respectively).

Multivariate analysis. In a first analysis, age, clinical stage and degree of differentiation were analysed. All these factors yielded significant, independent prognostic information (data not shown). In a second analysis, p53 was added to the mentioned variables (Table 1). Age and stage retained their significance and strong p53 expression was also strongly associated with poor survival prospects ($P < 0.01$). Differentiation yielded no additional prognostic information when p53 was included in the analysis.

In a third step, nuclear grade was added to the model (Table 2). Age and stage were still significant predictors of outcome ($P < 0.001$, respectively). Nuclear grade was also significantly associated with survival ($P < 0.01$). However, since p53 and nuclear grade were correlated, and nuclear grade was a stronger prognostic factor, p53 expression yielded no additive information when nuclear grade was included in the model.

In the last two analyses, flow cytometric data were evaluated. First, DNA ploidy was added to the aforementioned model (Table 3). Aneuploidy was prognostic in this model ($P < 0.05$), whereas nuclear grade lost much of its impact, since these two variables were highly correlated. Strong p53 expression yielded no significant extra information. After the inclusion of DNA ploidy, age and stage also lost some of their prognostic impact. In the last analysis, the S-phase fraction was included in its original continuous form. In this set of variables, aneuploidy was no longer significantly associated with survival, owing to covariation with the S-phase fraction (data not shown). In the models which included DNA and S-phase, the number of observations was reduced (frozen material for flow cytometry was not available in all cases), which partly explains the changes observed.

DISCUSSION

The main purpose of this investigation was to study the relationship between the expression of mutated p53 gene

Table 2. Multivariate analysis of clinical, histopathological variables, p53 and nuclear grade in patients in relation to disease-specific survival (n = 156, *events* = 66)

Variable	Categories	Relative hazard	95% confidence limits	*P* value
Age	Continuous	1.050	1.022–1.076	***
Clinical stage	I	1.0	Ref.	
	II	1.10	0.63–1.92	NS
	III	3.33	1.32–8.63	**
	IV	8.98	3.08–26.03	***
Differentiation	Well	1.0	Ref.	
	Moderate	0.84	0.41–1.69	NS
	Poor	0.86	0.37–1.96	NS
p53	No staining	1.0	Ref.	
	Weak or moderate staining	0.42	0.16–1.13	NS
	Strong staining	1.33	0.71–2.50	NS
Nuclear grade	1	1.0	Ref.	
	2	2.01	0.89–4.53	NS
	3	3.83	1.63–9.00	**

NS, non-significant; Ref, reference group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3. Multivariate analysis of clinical, histopathological variables, p53 and DNA content in patients in relation to disease-specific survival

Variable	Categories	Relative hazard	95% confidence limits	*P* value
Age	Continuous	1.034	1.003–1.064	*
Clinical stage	I	1.0	Ref.	
	II	0.93	0.51–1.70	NS
	III	2.72	0.97–7.59	*
	IV	7.86	2.60–23.72	***
Differentiation	Well	1.0	Ref.	
	Moderate	0.84	0.39–1.80	NS
	Poor	0.89	0.37–2.10	NS
p53	None	1.0	Ref.	
	Weak or moderate staining	0.24	0.07–0.86	*
	Strong staining	0.93	0.44–1.96	NS
Nuclear grade	1	1.0	Ref.	
	2	1.68	0.72–3.89	NS
	3	2.35	0.94–5.88	NS
DNA	< 2.3	1.0	Ref.	
	≥ 2.3	2.32	1.12–4.80	*

NS, non-significant; Ref, reference group; * $P < 0.05$; ** $P <= 0.01$; *** $P < 0.001$.

product and clinical and histopathological variables, and to evaluate the prognostic impact of p53 protein expression. In our material, a total of 45% of the samples were positive for p53 (27% were strongly stained), which is in agreement with previous studies [5, 15]. The distinction between weak and strong stainings was striking and only 5 cases were referred to the group of moderate p53 expression, although the interval for moderate expression was considerable (15–75%). Thus, the classification was easy to perform. Since the prognosis was strikingly much poorer for tumours that were strongly stained, this group was studied in detail.

Strong p53 expression was related to unfavourable histopathological subtypes, dedifferentiation, aneuploidy and high S-phase fractions. Thus, p53 expression was related to many high risk features, a finding that is supported by others [15, 16]. In the univariate analysis, survival prospects were much poorer in the strongly positive group than for the others. These results are in accordance with the sparse recent data from other studies of endometrial carcinomas [15–17]. Correlations between p53 and aggressive behaviour have also been demonstrated in several other types of human cancer such as ovarian cancer [4, 5], gastric and colorectal carcinomas [6], breast carcinomas [7] and bladder cancer [18].

In the multivariate analyses, p53 retained most of its prognostic value, even after adjustment for age, stage and differentiation. Thus, p53 expression is helpful in identifying high and low risk groups. However, when nuclear grade was added to the analysis (Table 2), p53 lost most of its independent prognostic value, since these two variables were highly correlated and nuclear grade was a stronger predictor of cancer-specific survival. The impact of nuclear grade in endometrial carcinoma has also been stressed by other authors [10, 19].

In agreement with other studies, DNA ploidy and S-phase were strong predictors of survival [14, 20, 21]. Since aneuploidy is a relatively objective marker of gross nuclear abnormalities, a high correlation between advanced nuclear grade and aneuploidy was not surprising. However, a relationship between a strong expression of mutated p53 protein and aneuploidy would also be expected. As elegantly described by Lane [22], 'wild type' p53 protein arrests damaged cells in G1, allowing repair or, if this is not possible, inducing apoptosis. The mutated p53 protein loses its inhibitory effect and, thus, cells with damaged DNA might undergo uninterrupted cell divisions, resulting either in mutations and aneuploidy or mitotic failure and cell death. Therefore, there is a natural relationship between a strong expression of mutated p53 protein, marked nuclear atypia and aneuploidy. Of these three variables, DNA ploidy was the strongest predictor of cancer-specific survival, and thus, p53 expression and nuclear grading added little prognostic information beyond DNA ploidy. These data are strongly supported by recent data of Lukes and associates [23].

It has been debated whether the immunohistochemical assessment of p53 mutations is of clinical interest, since false negative and false positive cases are known to exist and the commercial antibodies, such as DO-7, are not 100% specific. False negative cases might result if the point mutation does not stabilise the protein sufficiently, but then, the inactivation of the wild type protein is probably also incomplete [24]. Gross deletions might eradicate protein production but this rarely occurs. False positive cases are found more frequently, when the protein (with normally a half-life of 6–20 min) is stabilised for different reasons, without being mutated. This might, for example, be the case in tumour cells with a high level of mutant *RAS* oncogene expression [24]. Generally, associated cellular proteins might inactivate tumour suppressor activity of p53 by complexing to it. This is, for example, true for amplified MDM2 [22]. If a 'false positive' staining is indicative of a stabilised, inactivated (but not mutated) protein, it still gives some information about the functional status of the p53 pathway and might in such cases provide more prognostic information than DNA sequencing [24].

In conclusion, strong p53 protein expression is indicative of a poor prognosis in the univariate analysis and in the multivari-

ate analyses, which include age, stage and histopathological grade. Identification of mutated p53 protein expression also provides information about biological mechanisms in the tumour. However, if careful nuclear grading or DNA ploidy measurement are performed, p53 adds little extra prognostic information. Nuclear grading is easy to perform, it is inexpensive and can be done by any routine pathology laboratory and should therefore be used. DNA ploidy measurements are more objective and provide useful clinical information, beyond nuclear grade.

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