Overexpression of p53 protein is an independent prognostic indicator in human endometrial carcinoma

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Summary The important role of the p53 gene in tumour progression and cellular response to DNA damage has prompted investigation of the clinical significance of alterations to this gene. We examined both p53 overexpression and mutation of the gene in endometrial carcinoma in order to evaluate the prognostic significance of these changes. Of 122 endometrial carcinomas, 33 (27%) showed overexpression of p53 in the nucleus and 66 (54%) in the cytoplasm. Mutation in the p53 gene was found in 16 (13%) cases but showed no significant association with patient survival. Nuclear p53 overexpression was associated with poor survival (48% vs 80% alive in negative tumours 5 years post operatively, \( P < 0.001 \)). In contrast, cytoplasmic p53 overexpression was associated with better survival (85% vs 55%, \( P < 0.001 \)). When patients were separated into prognostic subgroups according to established clinical markers, these associations remained significant within most subgroups examined. In multivariate analysis adjusted for surgical stage, histological grade and type and vascular invasion, both nuclear p53 overexpression [hazard ratio 4.9 (95% CI 1.3–17.6), \( P = 0.016 \)] and cytoplasmic overexpression [0.25 (0.06–0.98), \( P = 0.047 \)] were independent prognostic factors. Immunohistochemical assessment of p53 overexpression in the nucleus and cytoplasm could provide useful prognostic information for the management of patients with endometrial cancer.

Keywords: p53; endometrial carcinoma; mutation; prognosis

Mutation of the p53 tumour-suppressor gene is the most frequently observed genetic alteration in human cancer (Levine et al., 1991). The p53 gene encodes a nuclear phosphoprotein that normally initiates G1 cell cycle arrest in response to DNA damage (Kuerbitz et al., 1992). This extends the time available for DNA repair and prevents genomic instability, hence the proposed role for p53 as a 'guardian of the genome' (Lane, 1992). In other situations, perhaps related to cell type or extent of DNA damage, wild-type p53 can also activate a form of programmed cell death known as apoptosis (Lowe et al., 1994). Recent evidence suggests that wild-type p53 is required for the apoptotic response of some tumours to chemotherapy and radiotherapy (Lowe et al., 1993, 1994). Because of its protective functions, p53 status may therefore be an important prognostic indicator for tumour response to adjuvant therapy and for overall patient survival.

Wild-type p53 protein is present at low levels in normal cells and is not usually visible using immunohistochemical (IHC) techniques. Mutation of the p53 gene in tumours can lead to stabilisation and accumulation of the protein so that it becomes readily visible by IHC techniques (Levine et al., 1991). It has been widely assumed that this represents mutant, inactive forms of the protein that can no longer carry out the protective functions of growth arrest and/or apoptosis in response to DNA damage. This may account for the association between nuclear p53 overexpression and worse prognosis observed in several previous studies on endometrial cancer (Inoue et al., 1994; Ito et al., 1994; Nielsen and Nyholm, 1994; Reinartz et al., 1994). The concordance between mutation and overexpression is not absolute however and the presence of a mutation cannot always be inferred from a positive IHC reaction and vice versa ( Wynford-Thomas, 1992).

In this study we examined the prognostic significance of both p53 overexpression and gene mutation in 122 endometrial carcinomas from patients with a long follow-up. Our results confirm that nuclear p53 overexpression is an independent prognostic indicator of shortened survival. We also report the novel observation of improved survival in cases with cytoplasmic accumulation of p53 protein.

Materials and methods

Specimens

Paraffin-embedded tissue blocks from 122 patients operated for primary endometrial carcinoma over the period 1979–87 were selected from the archives of King Edward Memorial Hospital for Women. An emphasis was placed on high-grade tumours and aggressive subtypes. Haematoxylin-and eosinstained sections were examined by a pathologist (KEW) to enable selection of blocks with maximal tumour content. No preoperative chemotherapy, hormonal therapy or irradiation was conducted before surgical excision of tumours. Histological classification was conducted according to WHO (Poulsen et al., 1975) and staging by the International Federation of Gynaecology and Obstetrics (FIGO) guidelines (Creasman, 1989). The histological subtypes were endometrioid (94 cases), serous papillary (14), clear cell (four), adenosquamous (three) and mucinous (seven). Other clinicopathological parameters such as peritoneal cytology, steroid receptor levels, myometrial invasion and surgical management were also recorded in each patient report. The median follow-up time was 60 months with a maximum of 165 months. At the end of the study period, 35 (29%) patients had died as a result of spread of their primary tumour.

Immunohistochemistry

Overexpression of p53 was detected using IHC as described previously (Dix et al., 1994) with prior antigen retrieval by microwave treatment (Gown, 1993). Two irrigations of 4 min each at 700 W in a 10 mmol l−1 citrate buffer (pH 6.0) were performed before blocking with 20% normal horse serum. Polyclonal anti-p53 CM-1 antibody (Novocastra Laboratories, UK), reported to recognise both wild-type and mutant p53 epitopes in formalin-fixed, paraffin-embedded tissue sections, was diluted at 1:1000 in 20%
normal horse serum/0.1% bovine serum albumin and incubated with sections for 18 h at room temperature. Positive controls were strongly staining colorectal tumour specimens identified in a previous investigation (Dix et al., 1994). These also served as negative controls by omission of the primary antibody during otherwise identical incubations. In addition, all cases were incubated with monoclonal antibody DO-7 (Novocastra Laboratories) at a dilution of 1:20 but using otherwise identical IHC conditions. Slides were evaluated independently for IHC staining by two pathologists (KEW and SK) who had no prior knowledge of clinicopathological features or patient outcome. Subcellular p53 localisation (nuclear and/or cytoplasmic) and the percentage of positive staining cells were recorded. Tumours with greater than 5% of malignant cells staining positive by IHC were considered to overexpress p53 protein.

Single-strand conformation polymorphism

DNA was extracted from paraffin-embedded tumour tissue as described previously (Sparrow et al., 1995) and used as template in polymerase chain reactions (PCRs) to amplify exons 5–8 inclusive of the p53 gene (Dix et al., 1994). Single-strand conformation polymorphism (SSCP) was then used to screen for mutations within the PCR products. Two different SSCP gel systems were used for the detection of mutations. [32P]dCTP-radiolabelled PCR product was denatured in formamide buffer (95% formamide, 10 mmol 1⁻¹ EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and the single-stranded DNA separated on 50 cm length polyacrylamide gels (12% acrylamide/10% glycerol, 1800 V, 18 h run) before visualisation of bands by autoradiography (Dix et al., 1994). The second SSCP gel system used was separation of non-isotopic PCR product on 8 cm length mini-gels (15% acrylamide/5% glycerol, 150 V, 5 h run) and visualisation of the single strands by silver staining. Gel staining consisted of 3 min in 10% ethanol, 3 min in 1% nitric acid, 10 min in 1% silver nitrate, development in a sodium carbonate (120 mg ml⁻¹) and formaldehyde (2.4 μl ml⁻¹) solution then fixation in 10% acetic acid for 5 min. All suspected mutations were confirmed by separate PCR and using both radioisotopic and non-radioisotopic SSCP gel methods. In preliminary experiments, we found the detection of mutations using both systems to be identical for 41 different p53 mutations (R Soong, in preparation).

Statistical analysis

For statistical analysis, prognostic parameters were treated as dichotomous variables as follows: FIGO stage (stage I/II vs III/IV), histological grade (grade 1/2 vs 3), myometrial invasion (less than one half vs greater than one half), histological type (endometrioid vs non-endometrioid). Patients whose primary cause of death was not recurrent endometrial cancer were censored from the study at the time of death. The chi-square method was used to determine the association between p53 alteration and other clinicopathological parameters. Fisher’s exact test was used when expected frequencies fell below five in any cell. The Kaplan–Meier method was used to construct survival curves for subgroups of patients. Comparison of curves was done using the log-rank test. Multivariate analysis was performed using Cox’s proportional hazards method. All analyses were conducted using the SPSS Software Package (Chicago, USA).

Results

Frequency of p53 alterations in endometrial carcinoma

Sections of endometrial cancers were stained with the polyclonal antibody CM-1 recognising wild-type and mutant forms of p53 protein (Midgley et al., 1992). IHC positivity was seen in both the nucleus (Figure 1a) and cytoplasm (Figure 1b) of tumour cells. Of the 122 cases examined, nuclear staining was observed in 27%, cytoplasmic staining in 54% and concomitant staining in both in 4% (Table I). Adjacent normal endometrial tissue was present in 53 cases. Of these, 12 (23%) showed very light cytoplasmic staining with CM-1.

Mutation of the p53 gene as detected by aberrantly migrating bands in SSCP gels (Figure 2) was observed in 13% (16/122) of endometrial tumours. Six mutations were found in exon 5, two in exon 6, eight in exon 7 and two in exon 8. Two of the tumours contained two different mutations. Of the tumours with exclusively nuclear staining, 9/28 (32%) contained a gene mutation, whereas 6/61 (10%) of exclusively cytoplasmic staining tumours contained a mutation.

Association of p53 alterations with clinicopathological features

Correlations between p53 alterations and the common clinicopathological prognostic indicators for endometrial cancer are summarised in Table I. Nuclear p53 overexpression associated significantly with the unfavourable prognostic indicators of advanced surgical stage, high-grade morphology, non-endometrioid histology, lymph node metastasis and vascular invasion, and the absence of progesterone receptor. In contrast, cytoplasmic p53 overexpression showed significant correlation with the favourable prognostic indicators of early surgical stage, low-grade morphology and absence of lymph node and vascular invasion. p53 mutations were associated with deep myometrial invasion, lymph node metastasis and positive peritoneal cytology.

Figure 1 Immunochemical detection of p53 protein in endometrial carcinoma with CM-1 antibody. (a) Nuclear overexpression. (b) Cytoplasmic overexpression. (Original magnification × 200).
histological grade ($P=0.034$), myometrial invasion 
($P<0.001$), peritoneal cytology ($P<0.001$) and lymph node 
($P<0.001$) and vascular invasion ($P<0.001$) were significant 
predictors of survival. Overexpression of nuclear p53 was 
associated with significantly worse prognosis ($P<0.001$; 
Figure 3b). Five year survival rates were 48% for patients 
with nuclear staining compared with 80% for those in which 
there was no nuclear positivity. In contrast, cytoplasmic 
staining correlated with improved survival ($P<0.001$; Figure 
3c): 85% compared with 55% at 5 year follow-up. Mutation of 
the p53 gene was associated with a trend towards worse 
survival (Figure 3d), but this was not statistically significant 
($P=0.115$).

Association of p53 alteration with survival was also 
examined within the more favourable prognostic subgroups 
of stage I/II, grade 1/2, endometrioid type and myometrial 
invasion of less than one half (Table II). Overexpression of 
nuclear p53 was associated with worse survival within all the 
subgroups examined except surgical staging whereas cyto-
plasmic overexpression was associated with better survival in 
all the subgroups. Interestingly, both p53 overexpression and 
mutation were significant predictors of survival in the 106 
patient subgroup treated post operatively with radiotherapy, 
but not in the 41 patients treated by chemotherapy.

### Multivariate analysis

Nuclear p53 overexpression was an independent prognostic 
indicator of survival in multivariate analysis adjusted for 
surgical staging, histological grade and type, vascular 
invasion and the interactions between overexpression and 
grade as well as grade and stage [hazard ratio 4.9 (95% CI 
1.3–17.6), $P=0.016$]. Cytoplasmic overexpression was also 
an independent prognostic indicator when adjusted for 
surgical staging, histological grade and type, vascular 
invasion and the interactions between overexpression and 
grade as well as overexpression and grade [0.25 (0.06–0.98), 
$P=0.047$]. The parameters of myometrial invasion, lymph 
node metastasis and peritoneal cytology are automatically 
accounted for in this analysis as they contribute to the 
surgical staging.

### Discussion

Recent advances in our understanding of the molecular basis 
of tumour development and response to treatment have 
opened up the possibility of finding novel and clinically useful 
prognostic indicators. In particular, the central role played by

### Table I: Correlation between p53 alterations and clinicopathological features

| Prognostic parameter (no. of patients) | Nuclear p53 overexpression (%) | Cytoplasmic p53 overexpression (%) | p53 mutation (%) |
|---------------------------------------|--------------------------------|----------------------------------|-----------------|
| Total (122)                           | 33 (27%)                      | 66 (54%)                        | 16 (13%)        |
| FIGO stage (122)                      |                               |                                 |                 |
| Stage I/II (91)                       | 19 (21%)                      | 56 (62%)                        | 9 (10%)         |
| Stage III/IV (31)                    | 14 (45%)                      | 10 (32%)                        | 7 (23%)         |
| (P = 0.009)                           | (P = 0.005)                   | (P = 0.119)                     |                 |
| Histological grade (122)             |                               |                                 |                 |
| Grade 1/2 (75)                       | 15 (20%)                      | 51 (68%)                        | 8 (11%)         |
| Grade 3 (47)                         | 18 (38%)                      | 15 (32%)                        | 8 (17%)         |
| (P = 0.027)                           | (P<0.001)                     | (P = 0.312)                     |                 |
| Histological type (122)              |                               |                                 |                 |
| Endometrioid (94)                    | 18 (19%)                      | 55 (58%)                        | 9 (10%)         |
| Non-endometrioid (28)                | 15 (54%)                      | 11 (39%)                        | 7 (25%)         |
| (P<0.001)                            | (P = 0.073)                   | (P = 0.052)                     |                 |
| Myometrial invasion (111)            |                               |                                 |                 |
| Invasion <0.5 (59)                   | 13 (22%)                      | 35 (59%)                        | 3 (5%)          |
| Invasion ≥0.5 (52)                   | 16 (31%)                      | 27 (52%)                        | 11 (21%)        |
| (P = 0.296)                          | (P = 0.433)                   | (P = 0.011)                     |                 |
| Peritoneal cytology (112)            |                               |                                 |                 |
| Negative (96)                        | 22 (23%)                      | 53 (55%)                        | 10 (10%)        |
| Positive (16)                        | 7 (44%)                       | 7 (44%)                         | 5 (31%)         |
| (P = 0.076)                          | (P = 0.395)                   | (P = 0.039)                     |                 |
| Lymph node invasion (97)             |                               |                                 |                 |
| Negative (78)                        | 14 (18%)                      | 52 (67%)                        | 8 (10%)         |
| Positive (19)                        | 10 (53%)                      | 4 (21%)                         | 6 (32%)         |
| (P = 0.006)                          | (P<0.001)                     | (P = 0.028)                     |                 |
| Vascular invasion (122)              |                               |                                 |                 |
| Negative (87)                        | 18 (22%)                      | 53 (61%)                        | 9 (10%)         |
| Positive (35)                        | 14 (40%)                      | 13 (37%)                        | 7 (20%)         |
| (P = 0.041)                          | (P = 0.017)                   | (P = 0.233)                     |                 |
| Progesterone receptor (67)           |                               |                                 |                 |
| Negative (16)                        | 8 (50%)                       | 6 (38%)                         | 4 (25%)         |
| Positive (51)                        | 10 (20%)                      | 29 (57%)                        | 3 (10%)         |
| (P = 0.025)                          | (P = 0.176)                   | (P = 0.201)                     |                 |
| Oestrogen receptor (87)              |                               |                                 |                 |
| Negative (13)                        | 6 (46%)                       | 6 (46%)                         | 2 (15%)         |
| Positive (74)                        | 18 (24%)                      | 39 (53%)                        | 10 (13%)        |
| (P = 0.174)                          | (P = 0.663)                   | (P>0.999)                       |                 |
| Age (122)                            |                               |                                 |                 |
| <66                                  | 17 (26%)                      | 34 (52%)                        | 10 (15%)        |
| ≥66                                  | 16 (29%)                      | 32 (57%)                        | 6 (11%)         |
| (P = 0.727)                          | (P = 0.534)                   | (P = 0.469)                     |                 |
abnormalities of p53 function in neoplasia has stimulated considerable interest in the use of alterations to this gene as potential markers of tumour behaviour. Owing to its technical simplicity most prognostic studies have used IHC to detect p53 overexpression, the assumption being that this represents mutant forms of the protein. In the present study we detected nuclear p53 overexpression in 27% of endometrial cancers using CM-1 antibody (Table I). A survey of the literature revealed an identical frequency of nuclear p53 overexpression (305/1150) in 13 previous IHC studies of this cancer type using a variety of other anti-p53 antibodies (Bur et al., 1992; Kohler et al., 1992; Jikko et al., 1993; Koshiyama et al., 1993; Ambros et al., 1994; Inoue et al., 1994a,b; Ito et al., 1994; Khalifa et al., 1994; Lukes et al., 1994; Nielsen and Nyholm, 1994; Reintartz et al., 1994; Schneider et al., 1994). We found nuclear p53 overexpression to be an independent prognostic marker of worse survival in endometrial cancer (Figure 3b). Although we have only presented data obtained using CM-1 polyclonal antibody, similar results were obtained with the DO-7 monoclonal antibody (not shown). Our findings confirm previous reports of an association between nuclear p53 overexpression and worse prognosis in endometrial cancer (Inoue et al., 1994b; Ito et al., 1994; Nielsen and Nyholm, 1994; Reintartz et al., 1994), however only the present study and that of Ito et al. (1994) found this to be an independent risk factor. Results from five separate studies using a variety of different anti-p53 antibodies appear therefore to provide conclusive evidence for an association between nuclear p53 overexpression and worse prognosis in this cancer type.

The major and novel observation from our work is that cytoplasmic p53 staining with CM-1 antibody is an independent prognostic indicator of improved survival in endometrial cancer (Figure 3c). With the exception of just two cases reported to show cytoplasmic staining in this cancer type (Inoue et al., 1994a), all previous studies have found exclusively nuclear staining using the anti-p53 antibodies 1801, DO-7 or DO-1 antibodies. In agreement with this we found only nuclear positivity when parallel sections were incubated with DO-7 (results not shown), suggesting the cytoplasmic staining we observed was due to the use of CM-1 antibody. A number of other investigators have shown cytoplasmic staining in a variety of cancer types including breast (Moll et al., 1992; Domagala et al., 1993; Stenmark-Askmalm et al., 1994), colorectal (Sun et al., 1992; Bosari et al., 1994), glioblastomas (Ali et al., 1994), undifferentiated neuroblastoma (Moll et al., 1995) and lung (Iggo et al., 1990). In one of these studies, Western blotting was used to confirm that CM-1 detected p53 in cytoplasmic-staining neuroblastomas (Moll et al., 1995). The biological significance of cytoplasmic p53 remains controversial however and the possibility of cross-reaction between CM-1 polyclonal antibody and one or more cytoplasmic proteins other than p53 cannot be excluded.

The few studies to date that have analysed cytoplasmic-staining tumours for p53 mutations, including our own, found normal allelotype in the large majority of cases (Moll et al., 1992, 1995; Ali et al., 1994; Bosari et al., 1995). Mutation of the nuclear localisation signals contained within the carboxyl-terminal domain (codons 290–393) was not found in cytoplasmic-staining breast tumours (Moll et al., 1992) or undifferentiated neuroblastomas (Moll et al., 1995).

Figure 2 PCR-SSCP detection of p53 gene mutation in endometrial cancers. Lanes 2 (sample E5) and 4 (E8) demonstrate the wild-type (WT) banding profile, while lanes 1 (E4), 3 (E7) and 5 (E12) contain additional, aberrantly migrating bands (arrows), indicating the presence of a gene mutation (M) in these specimens.

Figure 3 Kaplan–Meier analysis of survival association for (a) FIGO stage, (b) nuclear p53 overexpression, (c) cytoplasmic p53 overexpression, and (d) p53 gene mutation.
and hence is unlikely to account for the accumulation of p53 in this compartment. Instead it has been proposed that p53 is sequestered into the cytoplasm following binding to viral or cellular proteins and may therefore represent an alternative mechanism to mutation in causing functional inactivation of this gene in tumour cells (Moll et al., 1992). Another study suggests the intracellular distribution of p53 can be modulated by the conformation of the protein (Zerrahn et al., 1992). Adding to this are reports of cytoplasmic staining in normal breast epithelial cells (Moll et al., 1992; Takahashi and Suzuki, 1994), small hepatocytes (Zhao et al., 1994) and normal endometrium (present study), suggesting that it may also be a physiological phenomena.

In view of the uncertainty surrounding the nature of cytoplasmic staining with CM-1, it would seem premature to speculate on the basis of its association with improved survival in endometrial cancer patients. Similar to our findings, Moll et al. (1992) found a favourable prognosis for cytoplasmic-staining inflammatory breast carcinomas whereas the nuclear-staining tumours showed a worse outcome. In contrast to these results, two large investigations of colorectal cancer found an association between cytoplasmic CM-1 staining and worse prognosis (Sun et al., 1992; Bosari et al., 1994). It is difficult to reconcile the differences between these cancer types based on p53 mutation status alone as all the studies to date including our own indicate that cytoplasmic-staining tumours contain wild-type p53 (Moll et al., 1992; Ali et al., 1994; Bosari et al., 1995).

The present study is the first to analyse both p53 overexpression and gene mutation in a large series of endometrial cancers. Although the frequency of p53 mutation we observed was quite low (13%) in comparison with most other major cancer types, survey of the literature revealed an overall frequency of just 18% (54/298) for this cancer type (Okamoto et al., 1991; Risinger et al., 1992; Enomoto et al., 1993, 1995; Honda et al., 1993; Kohler et al., 1993; Schneider et al., 1994; Kihana et al., 1995). Our results show a trend towards worse prognosis for tumours with a p53 gene mutation (Figure 3d), however this did not reach significance (P = 0.11). With the exception of a recent report of poorer outcome for endometrial cancers with both p53 mutation and loss of heterozygosity (Kihana et al., 1995), patient numbers in previous studies have been too small to assess the prognostic significance of p53 mutations.

In conclusion, we found that nuclear and cytoplasmic p53 overexpression detected with CM-1 antibody were associated with worse and improved prognosis respectively in endometrial cancer. These correlations were independent of the established prognostic parameters of surgical stage, histological grade and type and vascular invasion. The differential staining was observed in a large proportion (73%) of endometrial cancers, raising the possibility of obtaining significant prognostic information from a single, routine IHC reaction. The additional information obtained may help to identify patients with early-stage and low-grade tumours who have a high risk of recurrence but are not detected using current clinical and pathological assessment procedures. These patients present the greatest difficulty in terms of planning the most appropriate treatment strategy. Overexpression of p53 in the nucleus and cytoplasm detected with the CM-1 antibody therefore has great potential as a novel prognostic indicator in endometrial cancer and future studies should aim to confirm our findings in larger retrospective and prospective studies of early-stage/low-grade tumours. 

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