Markers of the p53 pathway further refine molecular profiling in high-risk endometrial cancer: A TransPORTEC initiative

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HIGHLIGHTS

• We used markers of the p53 pathway to refine existing stratifications of endometrial cancer.
• We showed that mdm2 and p63 are useful markers to further classify this disease.
• This is particularly important in the copy number low tumours.

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ABSTRACT

Background. The morphological classification of high-risk endometrial cancer is of limited prognostic value. Recent attempts to stratify tumours according to molecular signatures have shown considerable promise. Here we attempted to further refine molecular classifications using markers of the p53 pathway.

Methods. We analysed the expression of p53 as well as three downstream markers of the p53 pathway, p21, mdm2 and phospho-p63 (pp63), by immunohistochemistry in a series of 114 endometrial cancers (86 endometrioid, 28 non-endometrioid subtype) with high-risk features (such as high tumour grade and deep myometrial invasion) and correlated results with clinical outcome. The Cancer Genome Atlas (TCGA) data were used to analyse TP63 mutations and copy-number alterations using cBioPortal. TP53 was silenced in two endometrial cancer cell lines to study its effect on p21 and pp63.

Results. About half of the tumours showed a p53 mutant phenotype and there was a strong negative correlation with p21 expression. Being marker positive for pp63 or mdm2 was associated with a significantly increased likelihood of dying, [hazard ratios 5.93 (95% CI 2.37–7.27) and 7.48 (95% CI 3.04–9.39), respectively]. These findings were seen in both p53 wildtype and p53 mutant tumours. Only 11% of TCGA endometrial cancers had a functional TP63 alteration. Upon silencing of TP53, p21 expression was decreased in one cell line, but no effects on pp63 were observed.

Conclusion. Markers of the p53 pathway improve stratification of endometrial cancers and provide novel insights into the role of this pathway in the disease.

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1. Introduction

The incidence of endometrial cancer is rising due to increased obesity and ageing of the population [1] and more women are dying from the disease as a result [2]. Overall survival following treatment for endometrial cancer is good, because most women present with low-risk disease. As the incidence of endometrial cancer increases, however, so too does the number of women with intermediate or high-risk disease.

The dualistic model of endometrial cancer, with type 1 and type 2 cancers, was first proposed over 30 years ago [3], and whilst it is still useful as an aetiological model of the disease, it is insufficiently discriminative to guide treatment decisions [4]. A Cochrane review based on five randomised clinical trials found a small but significant benefit for the addition of chemotherapy to adjuvant treatment for women with intermediate-high risk endometrial cancer [5] but significant toxicity is associated with this approach. The benefits were independent of histological subtype and therefore management is currently based on standard clinicopathological risk factors, including stage, tumour grade, depth of myometrial invasion and the presence of lymphovascular space involvement. The use of new stratification techniques to identify subgroups of women who are likely to benefit from adjuvant chemotherapy is an important area of unmet need [6].

More recent evidence suggests that molecular classification is more powerful than standard risk factors alone at identifying women at high risk of relapse who may benefit from adjuvant treatment. Four specific subgroups of EC were initially proposed by TCGA and have been subsequently confirmed within two large additional cohorts by the transPORTEC group [7,8] and the Canadian group that developed the slightly modified PromISE molecular classification system [9,10]. These studies have all described the emergence of four specific subgroups, albeit with slightly different nomenclature including the POLE mutative, mismatch repair deficient, copy number high/p53 mutant/p53 abnormal and copy number low/no specific molecular profile (NSMP)/p53 wildtype [7–9].

The largest single group identified in these series remains the copy number low or no specific mutation profile (NSMP) group. Given the variety of putative driver mutations identified within this group, such tumours are likely to be heterogeneous and may be suitable for further sub-stratification to aid further prognosis and prediction.

Mutation of the TP53 gene is a significant event in the evolution of many tumour types, including endometrial cancer. In general, p53 mutant status identifies a subgroup of patients with a poor prognosis but identifying aberrant p53 is not entirely straightforward. Most TP53 mutations result in protein stabilisation and overexpression of the protein, but some missense mutations result in a null phenotype leading to complete absence (or cytoplasmic staining) of p53 protein. The gold standard test for TP53 status is assumed to be mutational analysis but this technology is costly and can still lead to erroneous results. Moreover, p53 is a part of a complex pathway of proteins involved in recognition and management of DNA damage. Whilst p53 is the key protein in the pathway and perhaps the most commonly mutated in cancer, it is not the only protein to display aberrations or influence the functionality of the pathway. The identification of tumours with wildtype TP53 but altered p53 pathway function may have prognostic utility. The best described markers of this pathway are the downstream effector p21 and the repressor protein mdm2. In addition to these commonly studied markers, we investigated p63, a member of the p53 family.

 Immunohistochemistry analysis

Immunohistochemistry was conducted on tissue microarrays. Sections were dewaxed in histoclear and rehydrated in graduated alcohols. Following heat-induced epitope retrieval in citrate buffer (pH 6.0), endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Nonspecific binding sites were blocked with 20% blocking serum in TBS/0.1% Tween. Sections were incubated in primary antibody diluted in nonimmune blocking serum, in a humidified chamber overnight at 4 °C. p53, mdm2 and the phospho-p63 antibodies were used at 1:100 dilution, the phospho-p63 antibody being chosen as it detects both the TAp63 and the ΔNp63 isoforms. P21 antibody was used at a dilution of 1:250 (Supplementary table). The appropriate biotinylated secondary antibody (diluted 1:200) was added to each section for 1 h at room temperature. Antibody localization was carried out with the Vectastain Elite ABC Kit (Vector Laboratories) followed by incubation with 3,3-diaminobenzidine. Nuclei were stained with Mayer’s haematoxylin. As a negative control in all cases, primary antibody was replaced with normal immunoglobulin G (IgG) from the same species. Observers were blinded for patient characteristics and outcome. The slides were scored according to Table S1 (Supplementary materials), p53 was classified, as convention, into three groups based upon null, wildtype or overexpressing mutant phenotype [14] whilst all other markers were scored using a multiplicative nuclear intensity and area score [15] and then dichotomised around the median score.

2.3. Cell culture and gene silencing

The Ishikawa endometrial cell line was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). The HEC-1B endometrial cell line was maintained in DMEM and Hams F12 medium (1:1; Invitrogen) supplemented with 10% FBS. Cell lines were obtained from the European Collection of Cell Cultures and American Type Culture Collection, respectively. Both were authenticated at source by isoenzyme analysis and DNA profiling and continuously cultured for <6 months since authentication.
Cells were incubated at 37 °C in 5% CO₂. TP53 expression was reduced using a pool of 4 preselected siRNAs (FlexiTube GeneSolution, QIAGEN). siRNAs targeting TP53 were first diluted in Opti-MEM (Invitrogen) and then Lipofectamine 2000 reagent (Invitrogen). Transfection of cells was achieved by incubation with the Lipofectamine/siRNA mixture for 24 h at 37 °C and in 5% CO₂. After a further 24 h, cells were trypsinized, washed in medium, and harvested for protein extraction.

2.4. Western blotting

Nuclear protein was extracted from lysed cultured cells using the NE-PER nuclear and cytoplasmic extraction Kit (PIERCE Biotechnology) according to the manufacturer’s instructions. Protein content of the nuclear extract in the supernatant obtained was determined by a Bio-Rad protein assay according to manufacturer’s instructions (Bio-Rad Laboratories). Proteins (60 μg) were mixed 1:1 with loading buffer (22% glycerol, 139 mmol/L Tris-HCl, 154 mmol/L SDS, 4.4 mol/L urea, 0.002% bromophenol blue, and 10% vol/vol 2-mercaptoethanol) and heat-reduced for 5 min at 95 °C prior to separation on a 7% SDS-PAGE gel and electrophoretic transfer onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Nonspecific binding was blocked by incubating the nitrocellulose blots with 3% skimmed milk powder in TBS with 0.05% Tween. Blots were incubated with primary antibody (1:2000 with TBS/0.05% Tween) conjugated to HRP (Dako). Membranes were washed with TBS/0.05% Tween and protein bands visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech). The molecular mass of each visualized band was interpolated from a plot of log molecular mass versus distance migrated using kaleidoscope-prestained standards (Bio-Rad Laboratories).

2.5. TCGA analysis

Mutations, putative copy number alterations and mRNA (excluding miRNA, with z score set at 2 fold) were assessed for TP53 and TP63 in the TCGA 2013 endometrial carcinoma tumour set using the cBioPortal Analysis platform [16,17].

Table 1: Expression of p53 related proteins by histological subtype.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Total n</th>
<th>p53 n</th>
<th>p53 %</th>
<th>p21 n</th>
<th>p21 %</th>
<th>p63 n</th>
<th>p63 %</th>
<th>mdm2 n</th>
<th>mdm2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrioid</td>
<td>86</td>
<td>24</td>
<td>27.9</td>
<td>42</td>
<td>48.8</td>
<td>40</td>
<td>46.5</td>
<td>36</td>
<td>41.9</td>
</tr>
<tr>
<td>Serous</td>
<td>12</td>
<td>12</td>
<td>100.0</td>
<td>0</td>
<td>0.0</td>
<td>9</td>
<td>75.0</td>
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<td>44.4</td>
<td>16</td>
<td>88.9</td>
<td>15</td>
<td>83.3</td>
</tr>
</tbody>
</table>

Fig. 1. Representative images of endometrial cancer sections stained for p53 and downstream markers of the p53 pathway. p53 was classified into three groups based upon null, wildtype or overexpressing mutant phenotype whilst all other markers were dichotomised around the median score. Line represents scale bar measuring 100 μM. All images, unless stated, at ×10 magnification.
2.6. Statistics

All statistical testing was carried out in Prism v7. Kaplan Meier testing was used for overall survival analysis with logrank testing used to generate hazard ratios and 95% CIs and Mantel-Cox test for significance.

3. Results


The clinical characteristics of the patient cohort have been described previously and are reproduced in Table S3, Supplementary data. Previous regression analysis revealed only age as a prognostic factor for decreased recurrence-free survival (Supplementary data) [8].

3.2. Distribution of p53 markers

Samples were classified for p53 status as mutant (both the null and overexpression phenotypes) or wildtype. For all other markers scores were dichotomised around the median score, Fig. 1, Table 1. p53 was classified as mutant in 50/114 (44%) of tumours reflecting the high grade nature of the sample set and there was an inverse relationship with p21 expressing tumours [52/114 (46%)], Fig. 2. Mdm2 and phospho-p63 (pp63) were classified as high expression in 62/114 (54%) and 68/114 (59%) and showed strong correlation of staining with 59/71 (83%) of those samples showing high expression of one marker showing high expression both, Fig. 2. Twenty samples were classified as low expression for all markers.

3.3. The addition of markers of the p53 pathway improves stratification beyond p53 alone

Survival curves were generated for cases with survival data (n = 114). Cases with mutant p53 had lower overall survival than cases with wildtype (hazard ratio 4.9, 95% CI 2.7 to 8.8), Fig. 3a. Stratification by high expression of p21, mdm2 and pp63 also demonstrated marked survival differences, Fig. 3b–d. Showing higher expression for pp63 and mdm2 was associated with a significantly worse overall survival, compared to the negatives [hazard ratios 5.93 (95% CI 2.37–7.27) and 7.48 (95% CI 3.04–9.39), respectively], whilst showing high expression for p21 was associated with fewer deaths [hazard ratio 0.37 (95% CI 0.23–0.70)]. All of these associations were highly statistically significant (p < 0.005).

Cox Proportional Hazards Regression identified mdm2 as an independent prognostic factor [odds ratio 6.4 (95% CI 1.4–28.9)] whilst forward stepwise regression suggested that the combinations of either p53/mdm2 [odds ratio 5.2 (95% CI 2.3–12.0)] or p53/pp63 [odds ratio 4.3 (95% CI 1.9–9.7)] act as independent variables.

Combining pp63 with p53 expression identified a subgroup (p53 wildtype, pp63 negative), of 42 patients, that was associated with an exceptionally good overall survival. We therefore examined the overlap between this good prognostic group and the previously identified subgroups of POLE mutated and MMR deficient tumours [8], which were

![Fig. 2. Venn diagram showing interactions of p53 related proteins. Each ellipse represents mutant or positive expression respectively. Mdm2 expression was strongly correlated with pp63 expression. The single largest group of tumours (n = 36) were p53 mutant and expressed both mdm2 and pp63 but not p21. 20 samples were classified as negative for all markers.](image1)

![Fig. 3. Kaplan Meier curves for 114 cases of high-risk endometrial cancer showing overall survival stratified by expression of p53 and other downstream markers. All survival times in months.](image2)
similarly associated with a favourable prognosis. There was a strong association between p63 negative/p53 wildtype tumours and the POLE mutated phenotype with all but 4 of the POLE group also being p63 negative/p53 wildtype (p < 0.005, chi square), but there was no overlap with the MMR deficient (p = 0.1).

3.4. P63 expression is a poor prognostic marker in wildtype p53 tumours

Of the 114 cases analysed, 44 had been characterised as NSMP (no specific molecular profile) in our previous work [8]. None of these tumours had TP53 mutations or expressed abnormal p53 protein. We hypothesised that at least some of these tumours would have loss of function of the p53 pathway and would therefore be associated with poor outcomes. Stratification by p21 and pp63 of these NSMP tumours showed 25/44 (57%) exhibited high expression of pp63 which was associated with an increased risk of death [HR 5.5, (95% CI 2.1–14.2), p < 0.005]. No statistically significant difference was seen for tumours expressing p21, Fig. 4. We have previously demonstrated an association between TP53 mutation and L1CAM expression [18]. We therefore investigated the association between pp63 expression and L1CAM expression in p53 wildtype tumours, expecting to see similar co-expression, but found no such association (data not shown).

3.5. Silencing TP53 has no effect on protein expression of pp63

Ishikawa and HEC1B cells were transfected with siRNA to knockdown TP53 expression, Fig. 5a. Knockdown of TP53 in these two cell lines led to markedly different effects with regard to p21. In ishikawa cells which contain a M296V loss of function mutation in exon 7 [19], silencing of TP53 resulted in no change in p21 levels, whereas in HEC1B cells, which contain a R248Q, gain of function mutation in TP53 [20], there was reduced expression of p21. There was no change in the levels of pp63 in either cell line following knockdown of TP53.

3.6. TCGA analysis suggests TP63 genomic alteration is uncommon in endometrial cancer

The TCGA 2013 endometrial carcinoma database was used to explore the relationship between TP53 and TP63 mutations and copy-number alterations. The alteration rate of TP53 in the TCGA dataset was 67/232 (29%) with 50 missense, 14 truncating and 3 in-frame mutations. In contrast, the alteration rate of TP63 was just 26/232 (11%) with 14 amplifications, 2 deletions and 10 missense mutations. All 14 amplifications of TP63 occurred in tumours with mutant TP53 (p < 0.01, chi square test), whilst 11/12 deletion/mutation events occurred in wildtype TP53 tumours (p = ns, chi square test). The TP63 alterations found in serous cancers were exclusively amplifications, whilst the alterations in non-serous, endometrioid cancers were predominantly mutations or deletions. In keeping with our data there was no association in genomic and transcriptomic aberrations between p63 and L1CAM.

4. Discussion

In this study we have subjected a well-annotated set of endometrial cancers to immunohistochemical analysis of p53 pathway proteins. We show that use of these additional markers, in particular pp63, within the confines of this pilot study, refines stratification beyond the use of p53 alone by identifying subgroups with prognostic significance, and by distinguishing good and bad prognosis tumours within the otherwise unclassified “no specific mutation profile” group described previously [8].

These data suggest that pp63 expression may be associated with poor prognosis. The role of p63 in cancer biology remains poorly defined. P63 is a homologue of p53 that is indispensable for normal development. Like p53, it acts as a transcription factor and shares some commonality of target genes including p21 but notably does not act as a transcription factor for the death related genes Bax and PUMA [21]. Although it has been linked with both oncogenic and tumour suppressive functions in a variety of cancers [22], its properties may be related to the specific isoform expressed, with a shift towards expression of the ΔNp63α isoform in particular bestowing oncogenic potential [20]. The antibody used here identifies fractions of both major p63 isoforms but critically was a phospho specific antibody. This may partly explain why other studies which have used a total p63 antibody in a cohort of mixed risk endometrial cancer suggested that absent p63 expression, as opposed to the high or low as used in our study, was associated with reduced survival [19].

Our findings suggest that, in endometrioid-type endometrial cancer at least, p63 may be acting as an alternative to p53; pp63 is highly expressed in poor prognosis tumours. This is especially true in the wildtype p53 tumours and hints at the possibility of a gain of function phenotype analogous to that seen with mutant TP53, a phenomenon well described in developmental biology systems [23]. The association of pp63 expression with poor prognosis in a subset of endometrioid endometrial cancers is a novel finding. In other cancer types, including lung [12,24], p63 expression confers improved prognosis whilst for other tumour types, including gastric and cervix, the reverse is true. This may be a reflection of the tissue specificity of p63 including its well described role in squamous cell differentiation [21].

From our previous work and that of the TCGA, endometrial cancer subgroups associated with poor prognosis (p53) and good prognosis (POLE mutated and MMR deficient) have been described but residual groupings of intermediate risk disease remain. Indeed, the largest group of patients fall within NSMP and further refinement in this group is an urgent need to reduce over and under-treatment for these patients. To this end our finding that markers of the p53 pathway...
further sub-stratify the p53 wildtype NSMP group, is particularly interesting. Further planned work, within the context of the translational samples obtained from the PORTEC3 study [25], will elucidate this further and potentially identify any predictive role for these markers in this group.

It should be recognised that this work was limited to a cohort of high-grade tumours, that met the entry criteria for the PORTEC3 study. Further validation of our findings will be undertaken within the context of the PORTEC3 study once clinical data have matured, but the extrapolation of these findings to low-grade tumours should be avoided and further study of low-grade disease is justified. Nevertheless, our findings direct further study of the p53 pathway in intermediate-high risk endometrial cancer and define clinically useful prognostic groups for future validation.

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**Conflict of interest statement**

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2017.05.014.

**References**


