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Subclonal p53 immunostaining in the diagnosis of endometrial carcinoma molecular subtype

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Subclonal p53 immunostaining in the diagnosis of endometrial carcinoma molecular subtype

Aims: The significance of subclonal expression of p53 (abrupt transition from wild-type to mutant-pattern staining) is not well understood, and the arbitrary diagnostic cut-off of 10% between NSMP and p53abn molecular subtypes of endometrial carcinoma (EC) has not been critically assessed. Our aim was to characterise subclonal p53 and discrepant p53 expression/*TP53* sequencing results in EC and assess their clinical significance.

Methods and results: Subclonal p53 immuostaining on whole sections from 957 ECs was recorded. Agreement between *TP53* mutational assessment and p53 immunostaining was evaluated. Subclonal p53 IHC staining was seen in 4.0% (38 of 957) of cases, with 23 of 957 (2.4%) showing mutant-pattern p53 staining in ≥10% of tumour cells. It was most commonly seen in *POLE*mut (nine of 65, 14%) and MMRd (13 of 274, 4.7%) EC ('multiple classifier' ECs), where subclonal p53 staining does not impact the molecular subtype diagnosis. Excluding *POLE*mut and MMRd EC, 11 of 957 (1.1%) showed ≥10% subclonal p53 from which four patients died of disease, while there were no deaths due to disease in the five patients with <10% mutant-pattern p53 staining. Agreement between p53 immunostaining and *TP53* sequencing was 92.6%; most of the discrepant results were in the ultramutated *POLE*mut or hypermutated MMRd ECs. In NSMP and p53abn EC the agreement between IHC and sequencing was 95.8%.

Conclusions: Subclonal p53 staining ≥10% is present in only 1.1% of EC after excluding 'multiple classifier' ECs. The cut-off of ≥10% subclonal p53 staining identified patients at increased risk of dying from EC, supporting its use to diagnose p53abn molecular subtype.

Keywords: endometrial carcinoma, molecular subtype, p53 staining, subclonal staining, *TP53* mutation

Introduction

In the landmark 2013 study from The Cancer Genome Atlas (TCGA), four genomically distinct and prognostically significant endometrial carcinoma (EC) molecular subtypes were described: (i) *POLE* ultramutated, (ii) microsatellite instability hypermutated, (iii) copy-number low and (iv) copy-number high.[1] Pragmatic clinically applicable strategies were subsequently developed for diagnosis of the EC molecular subtype; these use targeted sequencing for pathogenic *POLE* mutations together with immunostaining for mismatch repair (MMR) and p53 to stratify cases into

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TCGA-analogous groups (i) *POLE* mutated (*POLE*mut), (ii) mismatch repair deficient (MMRd), (iii) no specific molecular profile (NSMP) and (iv) p53 abnormal (p53abn).[2–7] This approach has been validated and confirmed in multiple cohorts[2–7] and was endorsed by the World Health Organisation (WHO) in 2020,[8] and molecular subtyping is now integrated into prospective clinical trials to assess its ability to predict response to therapies.[9] Given what is known about the prognostic[2–7] and predictive[10,11] significance of EC molecular subtypes, implementation into routine clinical care, alongside traditional histopathological parameters, is progressing. This is due in large part to their incorporation into recent clinical management guidelines.[12–14]

One remaining challenge in molecular classification has been the interpretation and clinical implications of subclonal p53 staining. Subclonal p53 staining, defined as an abrupt transition between wild-type and mutant-pattern staining, is infrequently observed outside the context of *POLE*mut or MMRd, and its significance is not well understood.[15–17] WHO defines abnormal p53 staining as diffuse strong nuclear expression, complete absence of nuclear staining or cytoplasmic expression,[8] and 80% of positive tumour cells is often used as a cut-off. For subclonal mutant-pattern p53 staining, an arbitrary cut-off of ≥10% of tumour cells showing the abnormal staining pattern has been most commonly used to classify a tumour as p53abn,[16,17] but the clinical significance of this cut-off is unknown. There is known to be a low rate of discordance between p53 IHC/*TP53* sequencing with limited data to guide treatment decisions in these discrepant cases.[16,18] Our aim was to investigate the frequency, molecular basis and clinical significance of subclonal p53 immunostaining in EC and to correlate p53 staining patterns with *TP53* mutational analysis.

Materials and methods

CASE SELECTION

This study was approved by the University of British Columbia Institutional Research Ethics Board. Two cohorts were evaluated. The first was a national cohort of ECs diagnosed/treated in a single calendar year (2016) from the institutional archives of 29 participating Canadian centres.[19,20] There were 957 cases of 1357 in this study, where immunostaining for both p53 and MMR (PMS2 and MSH6) was performed centrally at Vancouver General Hospital and interpreted by the study pathologists. A second set of 10 cases with subclonal p53 staining (diagnosed/treated from 2012 to 2018) was provided by one of the authors (M.K.) from a single institution to use, together with 11 cases from the first cohort, for the microdissection studies. Clinicopathological and outcomes data were collected as described previously.[19,20] Cases (hysterectomy and/or biopsy specimens) were reviewed by pathologists at participating sites for the selection of best EC tumour block for molecular analyses.

HISTOMORPHOLOGICAL CLASSIFICATION

Histomorphological classification was based on surgical pathology reports, as described by Thompson *et al.*,[20] with central review conducted only in selected cases, as described below.

IMMUNOHISTOCHEMISTRY

Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE)-associated immunomarkers (p53, MSH6 and MSH2) were assessed on 4-μm thick whole sections cut and stained in the clinical laboratory at Vancouver General Hospital, using methods previously described.[19,20] Interpretation of immunohistochemistry (IHC) results was as follows: MMR IHC was recorded as intact or deficient; p53 IHC staining was classified as wild-type or abnormal (i.e. overexpression, null or cytoplasmic staining). The presence of any degree of subclonal p53 staining, defined as an abrupt transition between normal and mutant-pattern staining, was documented, including the % tumour cells on slides showing mutant-pattern staining. To ensure that true mutant-pattern p53 staining was present, a minimum size cut-off of a region or regions of contiguous tumour cells comprising at least 0.5% of the tumour cells on the slide was used for this study.

NEXT-GENERATION SEQUENCING

POLE mutation testing was performed by targeted next-generation sequencing (NGS) using methods previously described.[19,20] *POLE*mut assignment was limited to a list of 11 agreed-upon pathogenic mutations.[21] *TP53* mutational assessment was also undertaken in all cases. In cases showing subclonal staining, directed sequencing was performed on DNA extracted from cores or tumour scraped from slides in the areas with divergent immunostaining, i.e. wild-type and mutant-pattern staining, where tissue was available. To ensure sufficient representative tissue for NGS, the targeted sampling was limited to cases with

>5% of mutant-pattern subclonal staining. All *TP53* mutation calls were cross-referenced against publicly available databases (ClinVar, available at https://www.ncbi.nlm.nih.gov/clinvar/ and COSMIC, available at https://cancer.sanger.ac.uk/cosmic).[22,23] *TP53* mutational status for subclonal-stained cases underwent confirmatory sequencing with Sanger sequencing.

FINAL MOLECULAR SUBTYPE CLASSIFICATION

All ECs were classified into one of four molecular subtypes using the ProMisE and WHO endorsed algorithm, as reported previously.[19,20] If abnormal p53 subclonal staining was present in <10% of tumour cells they were considered to be p53 wild-type for purposes of molecular subtype diagnosis[10]; that is, if there was <10% mutant-pattern p53 staining in a *POLE* wild-type and MMR proficient tumour, it was considered NSMP. Cases with more than one molecular feature were classified in accordance with the segregation order and rationale described by León-Castillo *et al.*[17]

STATISTICAL METHODS

Univariable associations between molecular subtype and clinicopathological variables were evaluated as previously described.[19,20] Univariable associations between presence of subclonal staining and molecular subtype was undertaken. Agreement for *TP53* mutational status assessment between p53 immunostaining and NGS was evaluated in all cases and considering p53abn and NSMP ECs only (i.e. excluding the *POLE*mut and MMRd ECs). Kaplan–Meier analyses were used to assess the univariable effect of subclonal staining (uniform p53abn versus p53abn with subclonal staining of ≥10% versus p53 subclonal staining of <10%).

Results

COHORT DESCRIPTION

The clinical and pathological characteristics of the 957 patients with endometrial cancer from the national cohort are shown in in Table 1.

SUBCLONAL P53 IHC STAINING

Subclonal p53 staining (of any extent) was observed in 4.0% (38 of 957) of tumours, with 23 of 38 showing mutant-pattern p53 staining in ≥10% of tumour cells (Table 1, Table S1). The most common pattern of subclonal mutant-pattern staining was overexpression (29 of 38). The remaining nine tumours showing subclonal p53 staining included four with more than one mutant-pattern of p53 expression, two with subclonal-type p53 expression and three with subclonal cytoplasmic expression (Figure 1, Table 2).

The molecular subtype distribution of ECs with subclonal p53 staining (any extent) was as follows: nine *POLE*mut, 13 MMRd, 11 p53abn and five NSMP (based on subclonal staining in <10% of tumour cells and *POLE* wild-type/MMR-proficient) (Table 1). Of the 15 tumours where <10% of tumour cells showed subclonal mutant-pattern staining, one was *POLE*mut, nine were MMRd and five were NSMP (Table 2). Subclonal staining was most common in *POLE*mut EC (12.3%).

ASSOCIATION OF SUBCLONAL IHC STAINING AND MULTIPLE CLASSIFIER EC

Multiple classifier ECs, defined as tumours with more than one molecular feature, were observed in 3.0% (29 of 957) of cases and subclonal p53 expression, with ≥10% of tumour cells showing mutant-pattern p53 staining, was observed in 52% (12 of 23) of those multiple classifier ECs with mutant-pattern p53 staining (Table 3). Note that for the purposes of this analysis p53abn status was determined based on p53 immunostaining results. Using NGS to define multiple classifier tumours we would have identified 19 additional *POLE*mut EC with *TP53* mutations and 28 additional MMRd EC with *TP53* mutations (any VAF, Figure S1A). Those cases with discrepant p53 immunostaining and *TP53* sequencing results are considered separately below.

AGREEMENT OF P53 IHC WITH PRESENCE/ABSENCE OF *TP53* MUTATION

We first examined the correlation between p53 immunostaining and *TP53* mutational assessment in the 38 tumours showing subclonal expression of p53 by IHC; these results are shown in Table S1. A *TP53* mutation was identified in 31 of 38 cases (81.6%) showing subclonal p53 staining. *TP53* mutations were identified in 22 of 23 tumours with ≥10% subclonal staining, and in the one discrepant case without mutation detected, 98% of tumour cells showed null p53 staining, histology was serous and there was disease progression at 2.1 years. *TP53* mutations were less likely to be identified in tumours with <10% subclonal staining (22 of 23 versus nine of 15 in

Table 1. Characteristics for a national cohort of molecularly subtyped ECs where IHC staining for both p53 and MMRd were performed and reviewed centrally

Variable	Total		*POLE*mut		MMRd		NSMP		p53abn	
Total (*n*, % of cohort)	957	100.0%	65	6.8%	274	27.6%	473	49.4%	145	15.2%
Age at diagnosis, mean (years), SD										
≤60 years (*n*, % of cohort)	303	31.7%	41	63.1%	72	26.3%	172	36.4%	18	12.3%
>60 years (*n*, % of cohort)	654	68.3%	24	36.9%	202	73.7%	301	63.6%	127	87.6%
Histotype										
Endometrioid	784	84.2%	53	84.1%	255	95.9%	447	96.7%	29	20.5%
Low grade (grades 1, 2)	706	75.8%	44	69.8%	216	81.2%	433	93.7%	13	9.2%
High grade	78	8.4%	9	14.3%	39	14.7%	14	3.0%	16	11.3%
Non endometrioid	148	15.9%	10	15.5%	12	4.2%	14	3.2%	117	79.6%
Serous	67	7.2%	–	–	3	1.1%	2	0.4%	62	44.0%
Carcinosarcoma	32	3.4%	2	3.1%	1	0.4%	–	–	29	20.6%
Mixed carcinoma	22	2.4%	7	10.8%	3	1.1%	5	1.1%	7	5.0%
Clear cell	12	1.3%	–	–	–	–	5	1.1%	7	5.0%
De-diffferentiated/undifferentiated	3	0.3%	–	–	2	0.8%	0		1	0.7%
Other	12	1.3%	1	1.6%	2	0.8%	3	0.6%	6	4.3%
FIGO stage										
1A	563	61.3%	44	68.8%	150	57.3%	306	67.4%	44	68.8%
1B	190	20.7%	8	12.5%	64	24.4%	104	22.9%	8	12.5%
2–4	166	18.1%	12	18.8%	48	18.3%	44	9.7%	12	18.8%
Subclonal staining										
Subclonal p53 (any extent)	38	4.0%	9	13.8%	13	4.7%	5	1.1%	11	7.6%
Subclonal p53 (> 10%)	23	2.4%	8	12.3%	6	2.4%	0	0%	11	7.6%

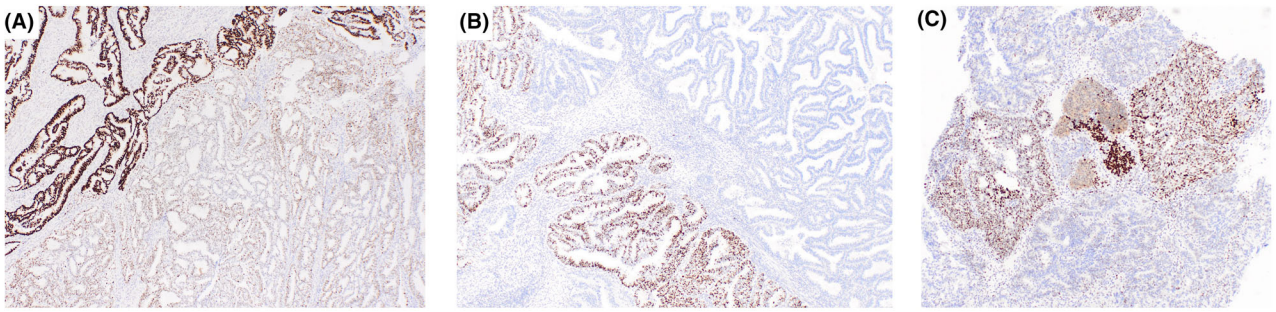

Figure 1. Subclonal p53 immunostaining patterns showing subclonal staining, defined as an abrupt change from wild-type to mutant-pattern staining. **A**, Subclonal p53 overexpression in a *POLE*mut tumour. **B**, Subclonal p53 null expression in a *POLE*mut tumour and **C**, subclonal p53 immunoexpression with multiple patterns of abnormal expression (null, overexpression and cytoplasmic expression, concurrent with complete MSH6 loss).

Table 2. Distribution of different subclonal p53 staining patterns across molecular subtypes

	Molecular subtype			
	*POLE*mut	MMRd	NSMP	p53abn
Subclonal staining				
≥10% overexpression	6	2		7
<10% overexpression	1	8	5	
≥10% null	2			
<10% null				
≥10% cytoplasmic		1		1
<10% cytoplasmic		1		
More than 1 mutant staining pattern		1		3
Total	9	13	5	11

Table 3. Multiple classifier ECs (i.e. more than one molecular feature)

	Wild-type p53 staining	Subclonal (≥10%) p53 staining	Diffuse abnormal p53 staining	Total
POLE/p53abn	0	7	4	11
POLE/MMRd	6	0	0	0
POLE/MMRd/p53abn	0	1	1	2
MMRd/p53abn	0	4	6	10
Total	6	12	11	29

Table 4. Comparison of p53 IHC and *TP53* mutation status in: (A) all tumours and (B), excluding MMRd and *POLE*mut tumours (p53abn and NSMP ECs only). Subclonal-stained cases (any extent) are not included in these analyses

(A)

		TP53 mutation	
		Present	Absent
p53 IHC	Abnormal	142	4
	Wild-type	64	709
Sensitivity: 68.9%			
Specificity: 99.4%			
Accuracy: 92.6%			
Positive predictive value: 97.3%			
Negative predictive value: 91.7%			

(B)

		TP53 mutation	
		Present	Absent
p53 IHC	Abnormal	131	4
	Wild-type	21	446
Sensitivity: 86.2%			
Specificity: 99.1%			
Accuracy: 95.8%			
Positive predictive value: 97.0%			
Negative predictive value: 95.8%			

≥10% and <10, respectively). This is an expected result, given that the cores for sequencing for this analysis were not directed; that is, they were taken from representative areas of tumour without intentionally sampling areas with mutant-pattern p53 immunostaining (as was conducted for the subset of cases described below).

In the remaining 919 tumours (excluding tumours with subclonal p53 staining), p53 immunostaining showed an accuracy of 92.6% [95% confidence interval (CI) = 90.7–97.2%] for the presence of a *TP53* mutation, with a sensitivity of 68.9% and a specificity of 99.4% (Table 4A). There were 64 tumours in which a *TP53* mutation was detected on NGS, but p53 IHC was interpreted as wild-type. Of these, 18 were *POLE*mut and 25 were MMRd ECs (Figure S1B). There were four tumours in which there was mutant-pattern p53 immunostaining and no *TP53* mutation detected on NGS; none of these four were *POLE*mut or MMRd EC. After excluding all MMRd and *POLE*mut tumours, the accuracy of p53 immunostaining increased to 95.8% (95% CI = 93.9–97.3%), with a sensitivity of 86.2% and specificity of 99.1% (Table 4B). Forty-three of 68 (63%) tumours with discrepant p53 immunostaining and *TP53* NGS results were either MMRd and *POLE*mut EC, where p53/*TP53* abnormalities are considered 'passenger mutations' and are not associated with an adverse prognosis.[17] Of the remaining 25 MMR-proficient and

POLE wild-type tumours, there were four with mutant-pattern p53 immunostaining in which no *TP53* mutation was detected. Of these, two showed null-type staining (one serous EC and one carcinosarcoma, both stage 3, with one patient dead of disease and the other patient alive and well after 3.5 years of follow-up), and two showed overexpression (one serous and one grade 1 endometrioid EC, both stage 1A, both alive and well after 1.7 and 4.5 years, respectively). Twenty-one EC were interpreted as showing p53 wild-type staining, but a *TP53* mutation was detected on NGS. Of these 21 cases, two had very low *TP53* variant allele frequency (<5%), one had both low allele frequency and the variant allele could not be independently verified by polymerase chain reaction (PCR) and one had a *POLE* mutation (Y458C) considered to be pathogenic in colorectal carcinoma,[24] together with three different *TP53* mutations and other mutations consistent with ultramutated phenotype. Of the remaining 17 tumours, most were low-grade endometrioid ECs (12 grade 1 and three grade 2), with one high-grade endometrioid, one serous and one clear cell EC. Of these 17 tumours with wild-type p53 immunostaining and *TP53* mutation on sequencing, all but two were stage 1, and there were two deaths due to disease within the first 2 years of follow-up (Table S2).

DIRECTED *TP53* SEQUENCING OF AREAS WITH WILD TYPE AND MUTANT-PATTERN STAINING

Twenty-one ECs with subclonal p53 IHC staining underwent directed *TP53* mutational analysis by NGS of areas of tumour showing wild-type and mutant-pattern p53 expression. Of these, nine showed at least one *TP53* mutation that was present in both the wild-type and mutant-pattern staining regions of the tumour, and these were considered to not be true subclonal mutations (Table 5). The 12 remaining tumours showed *TP53* mutations only in the abnormal staining region or there was a very significantly lower variant allele frequency (<10% VAF) in the wild-type staining area, and these were considered to represent true subclonal mutations (Table 5, Figure 2). Of the nine *POLE* mutated tumours, six had two or more *TP53* mutations detected on NGS, and two of the four MMRd EC had two *TP53* mutations.

OUTCOME ANALYSIS

Disease-specific survival of the 16 *POLE* wild-type and MMR proficient tumours, 11 of which had subclonal mutant-pattern staining in ≥10% of tumour cells (p53abn EC) and five had mutant-pattern p53 staining in <10% of tumour cells (NSMP EC), was compared to NSMP (n = 475) and p53abn (n = 145) EC with no subclonal staining (Figure 3). There were no disease-specific deaths in the patients with <10% subclonal p53 immunostaining and four disease-specific deaths among 11 patients with subclonal p53 expression in ≥10% of cells.

Discussion

In the new era of EC molecular classification, p53 IHC results and/or *TP53* mutation status is one of the critical molecular stratification features to assign molecular subtype, and impacts patient management. Having guidance on what should be called abnormal is now of great importance. According to the EC molecular classification algorithm,[8] p53 status is the last step in molecular assignment characterising EC patients without *POLE* mutations or MMRd to either p53abn or NSMP (assessed by either IHC or NGS). p53abn EC represent the most aggressive ECs, making up only 10%–15% of all ECs, but accounting for more than 50% of EC related mortality. Recent retrospective data have shown that identification of p53abn EC selects a group of patients who significantly benefit from adjuvant chemotherapy used in addition to radiotherapy.[10,11] The ESGO–ESTRO–ESP (2021) and ESMO (2022) EC guidelines now recommend that all p53abn ECs with myometrial invasion be considered high-risk and be treated with chemotherapy with or without radiotherapy, regardless of the stage, grade or histotype. In contrast, the majority of NSMP EC have an excellent prognosis, especially when low-grade and oestrogen receptor-positive, and treatment de-escalation can now be considered.[25,26]

p53 status can also have an effect on histotype assignment, as detection of mutant-pattern p53 staining favours high-grade diagnosis such as serous carcinoma or carcinosarcoma, although we now know that only 50% of p53abn ECs are serous carcinomas.[20,27] p53abn ECs are observed across a range of histotypes, including low-grade endometrioid ECs.

Subclonal p53 IHC staining is uncommon (4.0% of ECs with any extent, 2.4% with mutant-pattern p53 staining in ≥10% of tumour cells and only 1.1% of ECs after excluding 'multiple classifier' ECs). This is in line with previous work, although a higher frequency of subclonal staining was also seen in high-risk EC cohorts when lower cut-off points, such as 5% or 12 consecutive cells, were used.[16,18,28]

Table 5. Sequencing results obtained via directed sampling of areas with mutant pattern and wild-type pattern p53 staining

Study ID	Molecular subtype	% mutant pattern p53 staining on slide	Amino acid change	cDNA change	VAF in differentially staining areas (%)		True subclonal *TP53* mutation[†]
					p53abn	p53wt	
100	*POLE*mut	6	p.R306*	c.916 C>T	76.1	5.1	True
330	NSMP	8	p.H179R	c.536 A>G	28.6	Not seen	True
397	MMRd	9	p.Arg248Trp p.Pro278Thr	c.742C>T c.832C>A	30.8 22.5	45.1 75.3	Not true
147	*POLE*mut	11	p.R306* p.R282W	c.916 C>T c.844 C>T	9.7 14.8	Not seen	True
249	*POLE*mut	11	p.Y327* p.C238G	c.981T>G c.712T>G	14.9 12.4	Not seen	True
019	MMRd	11	p.P301QfsTer43	c.902delC	28.6	Not seen	True
072	*POLE*mut	15	p.R306* p.L289I p.R213* p.P152L	c.916 C>T c.865 C>A c.637 C>T c.455 C>T	22.6 10.7 37.7 16.5	Not seen	True
561	MMRd	15	p.R337C	c.1009 C>T	21.5	Not seen	True
256	*POLE*mut	20	p.R337C p.Y163H p.R213*	c.1009 C>T c.487T>C c.637 C>T	28.3 11.4 NA	Not seen Not seen 9.8	True
333	MMRd	50	p.R273C p.P301QfsTer43	c.817 C>T c.902delC	54.4 28.9	53.4 Not seen	Not true
439	*POLE*mut	50	p.R213* p.R213Q	c.637 C>T c.638G>A	68.3 Not seen	40.8 36.7	Not true
040	*POLE*mut	60	p.R213*	c.637 C>T	79.2	11.0	Not true
378	p53abn	60	p.S127P	c.379T>C	55.6	92.1	Not true
224	p53abn	65	p.G245S	c.733G>A	64.6	71.6	Not true
452	p53abn	65	p.V272E	c.815T>A	12.5	3.9	Not true
368	*POLE*mut	70	[intron 7] p.L130F	c.782+2T>G c.388 C>T	39.4 37.0	Not seen Not seen	True
271	p53abn	75	p.K132R	c.395G>A	89.5 +	4.9 −	True
609	*POLE*mut	85	p.R306*	c.916 C>T	76.1	5.1	True
229	p53abn	90	p.G245S	c.735 C>T	76.0	68.9	Not true
419	p53abn	95	p.Arg213*	c.637C>T	34.1	38.8	Not true
807	p53abn	95	p.R273H	c.818G>A	26.2	Not seen	True

All NGS results were validated by Sanger sequencing. 'Not seen' indicates mutations were was present in <5% VAF but not observed via Sanger sequencing.

+, Homozygous; −, heterozygous.

[†]Interpretation of the nature of the *TP53* mutations. True subclonal = mutation not present or present at significantly lower (<10%) VAF (probable contaminant) than in the mutated area frequency in the area of wild-type staining pattern of p53 IHC. Not true subclonal = same *TP53* mutation is present in both the IHC wild-type p53 and p53 abnormal components with at least 10% of the VAF.

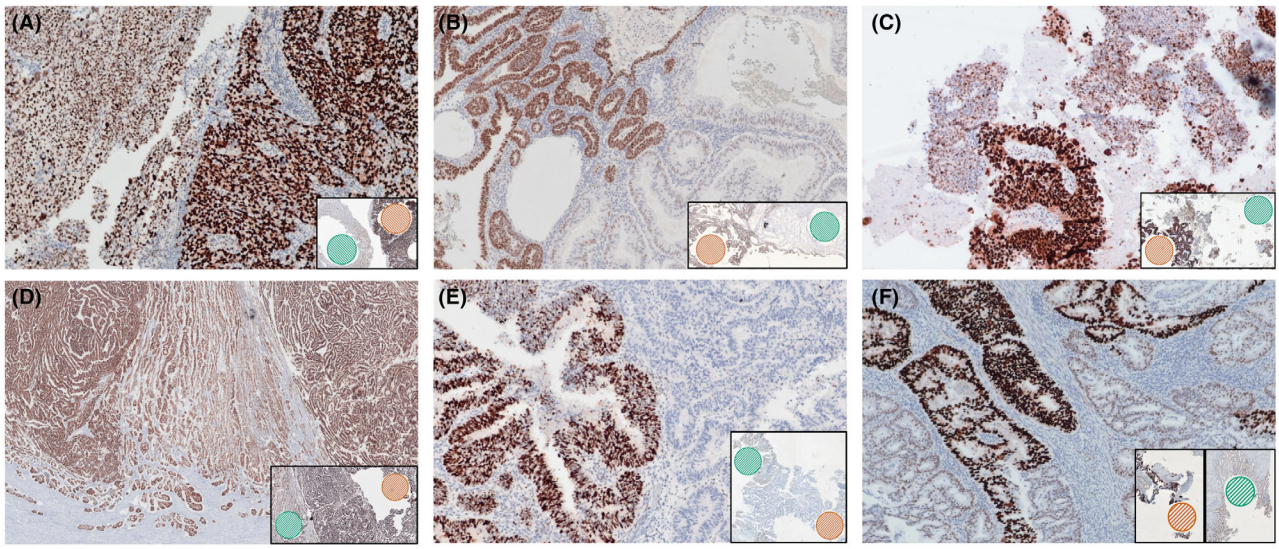

Figure 2. Selected cases that underwent directed sampling for next generation sequencing of *TP53*. Images depict post-core p53 IHC-stained slides; mutant-pattern and wild-type areas sampled for NGS are indicated by orange and green, respectively. **A**, A *POLE*mut-p53abn double classifier EC with 20% of tumour cells showing mutation-pattern p53 staining. Two mutations in *TP53* were present in the abnormal staining region and a third mutation was present in the wild-type staining region (ID 256). **B**, A p53abn EC with 60% of tumour cells showing mutant-pattern p53 staining. A *TP53* mutation was present in both the abnormal and wild-type-stained areas (ID 378). **C**, An MMRd-p53abn double classifier EC with 50% of tumour cells showing mutant-pattern p53 staining. Two *TP53* mutations were observed in both the abnormal and wild-type-stained areas (ID 333). **D**, A p53abn EC in which 75% of tumour cells show mutant-pattern p53 staining. A *TP53* mutation was present in both the abnormal (homozygous) and wild-type (heterozygous) staining areas (ID 271). **E**, A *POLE*mut-p53abn double classifier EC in which 50% of tumour cells show mutant-pattern p53 staining. A *TP53* mutation was observed in both the abnormal and wild-type staining areas (ID 439). **F**, An NSMP molecular subtype EC in which 8% of tumour cells show mutant-pattern p53 staining. A *TP53* mutation was observed in the abnormal staining area only (ID 330).

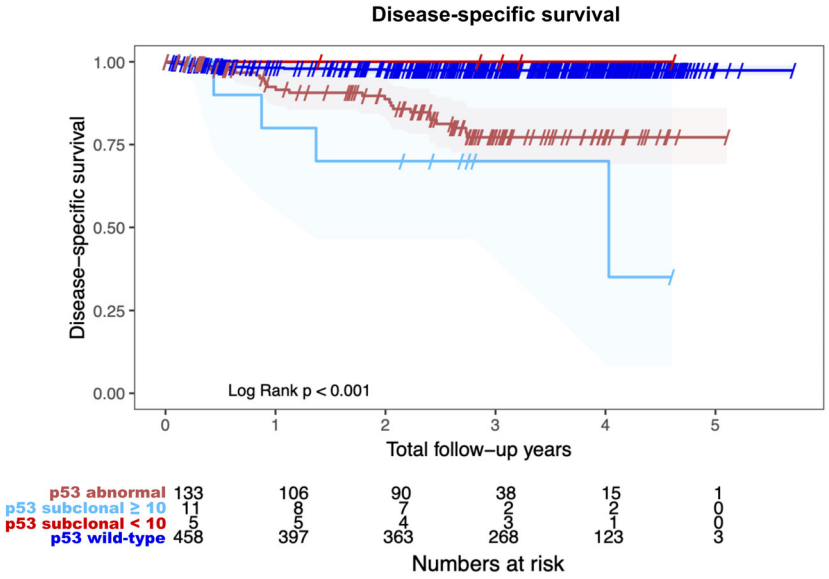

Figure 3. Kaplan–Meier curve of the prognostic significance of subclonal p53 immunohistochemical staining (<10 or ≥10%) on disease-specific survival in NSMP tumours compared to p53abn tumours (*POLE*mut and MMRd excluded).

When present, it is most commonly seen in *POLE*mut or MMRd EC ('multiple classifier' ECs), where subclonal p53 staining does not impact the molecular subtype diagnosis, and is thought to represent passenger mutations acquired during tumour progression in these ultramutated and hypermutated EC molecular

subtypes.[17] Only 16 of 957 (1.7%) of EC in this large cohort showed subclonal p53 mutant-pattern staining in the presence of wild-type *POLE* and proficient MMR, and in only 11 (11 of 957, 1.1%) of these was there subclonal staining in ≥10% of tumour cells. Because of the small number of cases with subclonal p53 staining, we were unable to adequately perform a critical determination of an optimal cut-off that is clinically relevant. It is reassuring, however, that in this series the arbitrary cut-off of 10% currently in use suffices to identify all disease-specific deaths.

In almost half of tumours (42.9%) with subclonal mutant-pattern staining for p53, the underlying *TP53* mutation is more widespread in the tumour than is appreciated based on the p53 immunostaining. The mechanism for wild-type staining in *TP53* mutated tumour cells was not explored, but could include variably altered regulation of p53 protein expression in tumour regions with *TP53* mutations, areas where there has not been loss of heterozygosity and deletion of the normal *TP53* allele, suboptimal fixation of tumour tissue or sampling issues, and merits further interrogation.

At this time, either p53 immunostaining or sequencing *TP53* can be used for molecular subtype diagnosis. Comparison of the two methods is critical, as there is insufficient evidence at present that they are equivalent. The accuracy of p53 IHC for predicting *TP53* mutations was 92.3% (sensitivity 68.8%) when all molecular subtypes were evaluated, and this improved to 95.8% (sensitivity 84.9%) when *POLE*mut and MMRd ECs were excluded from the analysis. This is similar to previous studies showing an overall accuracy of 91%–92% in all ECs, improving to 95% after excluding MMRd and *POLE*mut EC.[10,18]

In our series, only five tumours (including the one case with 98% null p53 staining) that had abnormal p53 immunostaining showed no *TP53* mutation on NGS. Three of five were of histotypes associated with p53abn molecular subtype (such as serous and carcinosarcoma) and three of five showed null p53 staining. False-negative results on NGS have been reported, but are fortunately rare and are often related to null type p53 staining commonly associated with large *TP53* deletions, which can remain undetected with NGS. If a *POLE* wild-type, MMR-proficient tumour has features suggestive of p53abn EC but without a *TP53* mutation on NGS, consideration should be given to performing p53 immunostaining. In 17 tumours a convincing *TP53* mutation was detected on NGS (VAF range = 5%–91%) but IHC showed wild-type staining. Most of these were low-grade endometrioid carcinomas, with two patients dying from disease within 2 years of treatment. Some of these *TP53* mutations are uncommon or of uncertain clinical significance, and together with the infrequent disease related events (recurrence and or disease-related death) in this group it is possible that sequencing may be too sensitive and IHC is a better surrogate marker of this aggressive molecular subtype. The significance of a *TP53* mutation in a *POLE* wild-type and MMR-proficient EC with wild-type p53 staining is, however, uncertain at this time and a larger series of such ECs with outcomes is needed to understand the clinical significance of this molecular phenotype. In a recent study of 164 ECs that underwent molecular classification, there was no discordance in p53 IHC versus *TP53* sequencing on NGS, suggesting that with advances in p53 IHC interpretation and attention to proper fixation of tumour tissue, this discordance is exceptionally rare.[29] In cases with discrepant NGS and IHC results, careful assessment of both *TP53* mutation and IHC should be performed, as either can give false negative results, and abnormality of either can be used to molecularly classify as p53abn molecular subtype.

Variant allele frequency is dependent upon tumour cell percentage submitted for NGS and cannot as such be used to define a 'subclonal' *TP53* mutation. With increasing use of NGS, which is required to determine *POLE* status, discordant p53 IHC and *TP53* NGS may be encountered more often and consensus on how to manage these discordant cases clinically will be important. Inconsistent molecular subtype assignment in these discordant cases could result in a significant difference in treatment recommendations, such as adjuvant chemoradiation if assigned p53abn versus surgery alone if assigned as NSMP EC.

In this series 3.0% of ECs were so-called 'multiple classifier' EC, having more than one molecular feature. It is noteworthy that most of the multiple classifier EC showed subclonal p53 staining. This follows from the observation, noted above, that most cases of EC with subclonal staining (22 of 38) were associated with a pathogenic mutation in *POLE* or MMR deficiency, thus the presence of subclonal p53 staining should prompt assessment of MMR expression and *POLE* sequencing, if not already performed.

Strengths of this study include the large sample size, with cases drawn from a wide range of clinical practice settings. It is the first study, to our knowledge, to critically assess the clinical and pathological correlates of subclonal p53 expression in EC and the currently used, but arbitrary, cut-off of 10% subclonal p53 expression as a diagnostic cut-off between NSMP and p53abn molecular subtypes of EC. Whole section immunostaining in an accredited diagnostic

laboratory was used to assess p53 expression. Weaknesses include the relatively small number of tumours with subclonal p53 expression, despite the large initial sample size, which precluded a rigorous assessment of the cut-off between NSMP and p53abn. Another weakness was non-standardised specimen handling and fixation, which could have impacted the immunostaining results.

In light of our work and others, our recommendations are that (i) cases with any extent of subclonal p53 staining should be tested for *POLE* mutations and MMR. Additionally, in *POLE* wild-type and MMR-proficient tumours, (ii) ≥10% of subclonal mutant-pattern p53 staining, defined as abrupt change from wild-type to mutant-pattern staining, justifies classification as p53abn, noting the presence (and extent) of subclonal staining, (iii) <10% of mutant-pattern staining in a NSMP should be commented upon in the report but the tumour is not classified as p53abn, and (iv) if subclonal p53 is identified on a biopsy specimen it should be repeated on a representative hysterectomy slide and that staining one representative slide is sufficient.

In conclusion, we demonstrate that subclonal mutant-pattern p53 immunostaining outside the context of MMRd or *POLE*mut EC, where it does not impact molecular subtype diagnosis, is rare and that the current cut-off of ≥10% mutant-pattern staining to diagnose p53abn EC serves to identify those patients with more aggressive disease.

Author contributions

EFT, CBG and JNM co-contributed to study conceptualization, data curation, investigation, methodology and project administration. JNM acquired study funding. JH, AJ and CBG contributed to formal analysis and writing of the original draft. SL provided data management and formal statistical analysis. AL, JS, JVB performed molecular studies and subsequent data analysis and validation. MK provided additional specimens. All co-authors contributed to manuscript review and editing.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability statement

Data available on request from the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *TP53* Boxplots showing the distribution of variant allele frequency (VAF) of TP53 mutation in (**A**) different molecular subtypes and (**B**) associated with different p53 IHC staining patterns, where a TP53 mutation was present.

Table S1. Mutations associated with subclonal p53 staining pattern and their variant allele frequencies.

Table S2. Details of tumours where a convincing *TP53* mutation was detected on NGS (VAF range 5%–91%) but immunohistochemistry showed wild type p53 staining.