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ORIGINAL PAPER

Use of mutation profiles to refine the classification of endometrial carcinomas

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Abstract

The classification of endometrial carcinomas is based on pathological assessment of tumour cell type; the different cell types (endometrioid, serous, carcinosarcoma, mixed, undifferentiated, and clear cell) are associated with distinct molecular alterations. This current classification system for high-grade subtypes, in particular the distinction between high-grade endometrioid (EEC-3) and serous carcinomas (ESC), is limited in its reproducibility and prognostic abilities. Therefore, a search for specific molecular classifiers to improve endometrial carcinoma subclassification is warranted. We performed target enrichment sequencing on 393 endometrial carcinomas from two large cohorts, sequencing exons from the following nine genes: *ARID1A, PPP2R1A, PTEN, PIK3CA, KRAS, CTNNB1, TP53, BRAF*, and *PPP2R5C*. Based on this gene panel, each endometrial carcinoma subtype shows a distinct mutation profile. EEC-3s have significantly different frequencies of *PTEN* and *TP53* mutations when compared to low-grade endometrioid carcinomas. ESCs and EEC-3s are distinct subtypes with significantly different frequencies of mutations in *PTEN, ARID1A, PPP2R1A, TP53*, and *CTNNB1*. From the mutation profiles, we were able to identify subtype outliers, ie cases diagnosed morphologically as one subtype but with a mutation profile suggestive of a different subtype. Careful review of these diagnostically challenging cases suggested that the original morphological classification was incorrect in most instances. The molecular profile of carcinosarcomas suggests two distinct mutation profiles for these tumours: endometrioid-type (*PTEN, PIK3CA, ARID1A, KRAS* mutations) and serous-type (*TP53* and *PPP2R1A* mutations). While this nine-gene panel does not allow for a purely molecularly based classification of endometrial carcinoma, it may prove useful as an adjunct to morphological classification and serve as an aid in the classification of problematic cases. If used in practice, it may lead to improved diagnostic reproducibility and may also serve to stratify patients for targeted therapeutics.

Keywords: endometrial carcinoma; uterine; mutation profiles; endometrioid; serous; carcinosarcoma; classification

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Introduction

The incidence of endometrial carcinoma is rising in the western world, and it is currently the most common type of gynaecological carcinoma [1]. This increase has been linked to increased obesity, increased life expectancy, and tamoxifen use in women [2]. The classical pathogenic dualistic model proposed by Bokhman in 1983 placed endometrial carcinomas into one of two groups: oestrogen-dependent endometrioid carcinomas and oestrogen-independent non-endometrioid carcinomas [3]. The classification of endometrial carcinomas used in clinical practice is based on histopathological assessment to determine cell type and grade [4,5], and is used in guiding therapy [6,7]. Endometrioid endometrial carcinomas (EECs) represent 70–80% of cases, are generally low grade (grade 1 or 2) with a favourable prognosis, and most are cured by hysterectomy alone [8,9]. However, less common high-grade (grade 3) endometrioid carcinomas (EEC-3s) have a significantly worse prognosis [5,10]. The remaining 20–30% of non-endometrioid subtypes consist mostly of serous, and less commonly carcinosarcoma (previously known as MMMT or mixed malignant Müllerian tumours), mixed histology, undifferentiated, and clear cell carcinomas. These non-endometrioid tumours are not generally graded in the WHO grading system [6] and are considered high grade, as they are associated with poor outcomes [11]. Recent reports have shown that the current pathological classification and grading system of high-grade endometrial carcinomas is limited in both reproducibility and prognostic ability [10,12–14].

Molecular alterations in the PI3K/AKT, MAPK, and WNT signalling pathways have been implicated in the pathogenesis of specific endometrial carcinoma subtypes [15–18]. Thus, there is a rationale for using mutational profiles in the classification of these tumours. EECs are molecularly recognized by frequent mutations in *PTEN, PIK3CA, KRAS, CTNNB1*, *FGFR2*, and microsatellite instability (MSI) [8,19–22]. Recent studies have identified mutations in *ARID1A* [34], *PIK3R1* [23], and *PIK3R2* [24] in EECs. Endometrial serous carcinomas (ESCs) and carcinosarcomas characteristically do not harbour a high frequency of these mutations; however, *TP53* [8,19,20], and *PPP2R1A* [25,26] mutations are known to be common in ESC. *TP53* mutations are also detected in carcinosarcomas [27] and EEC-3s [10,28].

Next-generation sequencing technologies have allowed the sequencing of multiple genes and samples simultaneously [24], making large mutational studies achievable. As no single gene is a sensitive or specific marker for endometrial carcinoma subtypes, it is likely that the analysis of gene panels will be needed to guide subclassification. The aim of this study was to determine the mutation profiles of a large series of endometrial carcinomas, based on oncogenes and tumour suppressor genes known to be important in carcinogenesis, in an attempt to improve the classification of endometrial carcinomas.

Materials and methods

Tumour samples

We obtained 152 endometrial tumours and 90 corresponding buffy coat specimens originating from the BC Cancer Agency and Vancouver General Hospital via the OvCaRe Tissue Biobank Repository, Vancouver, BC, Canada. Patients were informed for written consent, and research ethics approved as previously described [25]. An additional 260 endometrial tumour DNA samples were obtained from Washington University, St Louis, Missouri, USA. The endometrial subtype, grade, and microsatellite instability data were previously determined. All samples from both centres had undergone review by gynaecological pathologists. The EEC-3 cases were diagnosed as grade 3 based on more than 50% solid architecture in all but four cases, where the diagnosis of grade 3 was based on the combination of grade 2 architecture and notable nuclear atypia. A small number of clear cell carcinomas were specifically excluded in this analysis, as slides were not available to confirm the diagnosis in these cases.

Exon sequencing

Genomic DNA (500 ng) was used for indexed Illumina library construction [29] and then underwent targeted enrichment using biotinylated RNA capture probes generated from cDNA clones or PCR amplicons [30] representing exons of *ARID1A, PTEN, PIK3CA, KRAS, CTNNB1, PPP2R1A, BRAF, TP53*, and *PPP2R5C* and sequenced using Illumina (GAIIx).

Bioinformatics analysis

Short reads were aligned to the human genome (hg18) using the BWA aligner v0.5.9 [31]. A Random Forest classifier trained on validated SNVs was used to remove false-positive calls [32]. SNVs in the Catalogue of Somatic Mutations in Cancer (COSMIC) [33] were considered to be true positives, so a 99% cut-off threshold was selected (Supplementary Figure 1). Mean coverage was plotted for cases with and without mutations (Supplementary Figure 2). Details may be found in the Supplementary materials and methods.

DNA validations

Select predicted SNVs were validated using Sanger sequencing as previously described [25] (see the Supplementary materials and methods).

Identifying outlier cases

Outliers were identified by observing mutation profiles that did not fit the original diagnosed histological subtype—defined as ESC with *PTEN* and/or *ARID1A* mutations, and low-grade EECs with only *TP53* and/or *PPP2R1A* mutations. With the goal of comparing mutational outliers with immuno-profiles, formalin-fixed, paraffin-embedded blocks were available for

only 147/156 Vancouver cases for the construction of a tissue microarray (TMA). (Details may be found in the Supplementary materials and methods.) These cases were used for the characterization of mutational outliers, by correlating with morphology and immunohistochemistry (IHC), and were retrospectively reviewed by two independent pathologists, using the full hysterectomy case, without knowledge of mutation or IHC data.

Statistical analysis

Fisher exact tests and multivariable logistic regression analysis were used to test the significance of associations between mutations within subtypes. All tests were two-tailed and p values less than 0.05 were considered significant. Fisher exact tests were used to perform exploratory analyses; accordingly, no multiple comparison corrections were performed. The multivariable logistic regression model used step-wise selection based on the likelihood ratio test, with all genes included. The Hosmer–Lemeshow test was used to assess the goodness-of-fit of the estimated logistic regression models.

Results

To determine the mutation frequencies in various subtypes of endometrial carcinomas, we used exon capture sequencing of *ARID1A, PTEN, PIK3CA, KRAS, CTNNB1, PPP2R1A, BRAF, TP53*, and *PPP2R5C*. This resulted in the detection of somatic non-synonymous missense, truncating indels (insertions/deletions), and splice site mutations in 90.1% (353/392) of cases. The characteristics of the endometrial carcinomas, with histology subtypes and grade, are summarized in Table 1. We have stratified these carcinomas into low-grade (grades 1 and 2) EECs, EEC-3, ESC, carcinosarcoma, mixed, and undifferentiated, based on routine histopathological assessment, to determine the differences in mutational profiles. All mutational data are summarized in Supplementary Table 1. The mutation frequencies of *ARID1A, PTEN, PIK3CA, PPP2R1A, TP53*, and *CTNNB1* are significantly different across four subtypes of endometrial carcinomas (Table 2).

High-grade and low-grade endometrioid carcinomas have similar mutation profiles but differ in the frequencies of *TP53* mutations

Low-grade EECs have high to moderate frequencies of mutations in *PTEN, ARID1A, PIK3CA*, and *CTNNB1* (Table 2), with a higher frequency of mutations of *PTEN, ARID1A, PIK3CA, KRAS, PPP2R1A*, and *TP53* seen in EEC-3s (Table 2). Comparison of the mutations in low-grade EEC and EEC-3 showed that *PTEN* ($p = 0.0111$) and *TP53* ($p = 0.0046$) mutation frequencies are significantly different (Table 3). Multivariable logistic regression also revealed that *PTEN* ($p = 0.007$) and *TP53* ($p < 0.001$) mutations significantly distinguish EEC-3 from low-grade EEC (Table 4).

Table 1. Summary of all endometrial carcinoma subtypes

All subtypes	
Endometrioid	306
Grade 1	169
Grade 2	107
Grade 3	30
Serous	37
Mixed*	4
Undifferentiated	3
Carcinosarcoma	42
Total	392

*Includes one case as mixed serous and endometrioid carcinoma, one case mixed G2 and G3 endometrioid and clear cell carcinoma, and two cases as mixed serous and clear cell carcinoma.

Endometrial serous carcinomas show a distinct mutation profile

Of 37 ESCs, high frequencies of mutations were found in *TP53, PPP2R1A*, and *PIK3CA* (Table 2). *TP53* and/or *PPP2R1A* mutations were found in 28/37 (75.7%) ESCs, accounting for the majority of aberrations in this subtype (Figure 1). Comparison of EEC-3 to ESC revealed significantly different mutation frequencies for *ARID1A, PTEN, PIK3CA, CTNNB1, PPP2R1A*, and *TP53* ($p < 0.05$) (Table 3). Low frequencies to zero mutation events were noted for some genes common in both ESCs and EEC-3. In an attempt to keep all the multivariate analyses consistent across the subtype comparisons, we included the same list of genes in the logistic regression model building between EEC-3 and ESC. As a result, there was no one reliable multivariable logistic regression model built, based on the mutation markers, to distinguish between these two subtypes (Table 4). As expected, the mutational profiles of low-grade EEC and ESC were significantly different (Table 3). Multivariable logistic regression showed *PTEN* ($p < 0.001$) with a trend of *ARID1A* ($p = 0.08$) mutations associated with low-grade EEC, whereas *PPP2R1A* and *TP53* ($p < 0.001$) were associated with ESC (Table 4).

Cases with discordant morphological diagnosis and mutational profiles

As discussed, ESCs were found to have a high frequency of mutations in *TP53* and *PPP2R1A* (Figure 1). From the mutation profiles, we identified three histology-defined ESC cases with *ARID1A* and *PTEN* mutations but lacking *TP53* mutations, a profile more indicative of EECs (Figure 1). Other studies have not found *ARID1A* or *PTEN* mutations in ESCs; however, there have been limited studies testing for *ARID1A* mutations in endometrial carcinomas [34–36]. On independent histopathological review of these three cases, all were mixed tumours consisting predominantly of ESC, but with minor components of low-grade EEC in two cases and EEC-3 with clear cell

Table 2. The frequency of mutations within all endometrial subtypes

	Low-Grade Endometrioid (G1 and G2) ($n = 276$)	High-Grade Endometrioid (G3) ($n = 30$)	Serous ($n = 37$)	Carcinosarcoma ($n = 42$)	p value across all subtypes (chi-squared test)
PTEN	185 (67.0%)	27 (90.0%)	1 (2.7%)	14 (33.3%)	**4.63E-17**
PIK3CA	105 (38.0%)	17 (56.7%)	10 (27.0%)	12 (28.6%)	**0.0480**
ARID1A	129 (46.7%)	18 (60.0%)	4 (10.8%)	10 (23.8%)	**5.8E-06**
KRAS	46 (16.6%)	8 (26.7%)	3 (8.1%)	7 (16.7%)	0.24
CTNNB1	66 (23.8%)	6 (20.0%)	1 (2.7%)	2 (4.8%)	**1.2E-03**
PPP2R1A	19 (6.9%)	3 (10.0%)	16 (43.2%)	9 (21.4%)	**1.5E-09**
TP53	28 (10.1%)	9 (30.0%)	25 (67.6%)	27 (64.3%)	**2.8E-23**
BRAF	8 (2.9%)	2 (6.7%)	2 (5.4%)	1 (2.4%)	0.62
PPP2R5C	1 (0.4%)	2 (6.7%)	0 (0%)	0 (0%)	**0.002**

Bold indicates significant p-values <0.05.

Table 3. Univariate Fisher exact test (p-values) to show significant differences between mutation profiles of each endometrial carcinoma subtypes

	Low-Grade Endometrioid vs High-Grade Endometrioid	Low-Grade Endometrioid vs Serous	High-Grade Endometrioid vs Serous	High-Grade Endometrioid vs Carcinosarcoma	Serous vs Carcinosarcoma
PTEN	**0.01**	**6.6E-15**	**2.6E-14**	**1.1E-06**	**4.3E-04**
PIK3CA	0.052	0.21	**0.024**	**0.028**	1.00
ARID1A	0.18	**1.4E-05**	**2.4E-05**	**0.003**	0.15
KRAS	0.20	0.23	0.053	0.38	0.32
CTNNB1	0.82	**1.2E-03**	**0.039**	0.06	1.00
PPP2R1A	0.46	**5.0E-08**	**3.0E-03**	0.34	0.053
TP53	**4.6E-03**	**8.6E-14**	**3.2E-03**	**0.008**	0.82
BRAF	0.26	0.34	1.00	0.57	0.60
PPP2R5C	**0.026**	1.00	0.20	0.17	NA

Bold indicates significant p-values <0.05.

carcinoma in one case (Table 5). For the two mixed ESC and low-grade EEC cases, we confirmed the section of tumour sample used for DNA extraction and subsequent sequencing exclusively contained the ESC component (Figure 2); however, it harboured mutations with an endometrioid profile. Immunostaining is recommended for use in diagnostically problematic cases [37], although not universally used. These three cases showed a non-serous IHC profile, p53 normal expression, and p16-negative expression, while one expressed ER and PR (Table 5).

We also identified four outlier low-grade EECs that contained *TP53* mutations and lacked *PTEN* mutations, which were also diagnostically challenging cases. Upon review, two cases showed morphological features of serous carcinoma, and one case was re-classified from low-grade EEC to EEC-3. One outlier remained classified as low-grade EEC; however, it was noted that this case showed extensive myometrial invasion and widespread lymphovascular invasion. By IHC, abnormal p53 expression was confirmed in all cases. All were, however, ER-positive with PTEN loss of expression, features found primarily in EECs. In two of these cases, p16 was strongly expressed (Table 5). In summary, these seven outlier cases showed features intermediate between ESC and EEC in morphological, IHC, and genetic analysis (Table 5 and Supplementary Table 2).

We also performed unsupervised hierarchical clustering analysis on the 147 cases with IHC and mutational status (Supplementary Figure 3 and Supplementary Table 2). This showed that most low-grade EEC and EEC-3 subtypes clustered together, while the remaining EEC-3, serous and mixed cases were scattered. The mutational outliers with the diagnosed subtype are indicated, as well as the new classification.

Carcinosarcomas show either an endometrioid or a serous mutation profile

Endometrial carcinosarcomas are relatively rare, and their classification as an endometrial carcinoma subtype or as a distinct entity is under debate [38]. In our analysis of carcinosarcomas, we found mutations in *TP53, PTEN, PIK3CA, ARID1A*, and *PPP2R1A* (Table 2). Two subgroups of carcinosarcomas were identified: one group characterized by mutations in *PTEN* and *ARID1A* (endometrioid type), and a second group with *TP53* and *PPP2R1A* mutations more similar to ESC (Figure 1). Heterologous differentiation of the sarcomatous component was observed in a subset of tumours from both groups. Histopathological reviews of cases were not available; therefore it was not possible to correlate morphological features and mutational profiles of endometrioid-like or serous-like in the epithelial components of these tumours.

Mutations involving signalling pathways in endometrial carcinomas

By mutational analysis of multiple genes, it is possible to identify different mutations involving a single

Table 4. Multivariable logistic regression analysis of gene mutations between endometrial carcinoma subtypes. Reported values are only the most significant genes selected by the step-wise selection method based on the likelihood ratio test

Gene (marker)	Low-grade endometrioid ($n = 276$) vs high-grade endometrioid ($n = 30$)		Low-grade endometrioid ($n = 276$) vs serous ($n = 37$)		High-grade endometrioid ($n = 30$) vs serous ($n = 37$)		High-grade endometrioid ($n = 30$) vs carcinosarcoma ($n = 42$)		Serous ($n = 37$) vs carcinosarcoma ($n = 42$)	
	p value	OR* to high-grade endometrioid	*p* value	OR* to serous	*p* value	OR* to serous	*p* value	OR* to carinosarcoma	*p* value	OR* to carinosarcoma
PTEN	0.007	5.61 (1.6–19.7)	1.89E-04	0.02 (0.002–0.14)			3.75E-05	0.05 (0.01–0.22)	6.24E-03	19.41 (2.3–162.6)
PIK3CA										
ARID1A			0.080	0.3 (0.08–1.2)						
KRAS										
CTNNB1										
PPP2R1A			2.7E-04	13.28 (3.3–53.4)			0.0736	5.12 (0.86–30.7)	0.0446	0.32 (0.1–0.97)
TP53	7.04E-04	4.95 (2.0–12.5)	7.64E-05	7.64 (2.8–20.9)						
BRAF			1.40E-02	18.9 (1.8–196.7)						
PPP2R5C										

*OR = odds ratio (95% confidence interval).

signalling pathway that may be functionally equivalent, and to examine the relationship between mutations involving different genes/pathways. Mutations in the PI3K and MAPK signalling pathways are known to be important in EECs; therefore we further examined the prevalence of mutations in *PTEN, PIK3CA, KRAS, ARID1A*, and *CTNNB1*. We found that 211/276 (76.5%) low-grade EECs had *PTEN* and/or *PIK3CA* mutations (Figure 1). Co-existent *PTEN* and *PIK3CA* mutations were identified in 79/276 (28.6%) low-grade EECs and 16/30 (53.3%) EEC-3s ($p = 0.0112$). *AR1D1A* mutations have recently been identified in low-grade EECs; however, the relationship of these mutations with other pathways such as PI3K and WNT has not been examined [34]. Of the low-grade EECs with *ARID1A* mutations, 112/129 (86.8%) had mutations within *PTEN* and/or *PIK3CA* ($p = 0.0002$). EEC-3s with *ARID1A* mutations ($n = 18$) all had *PTEN* mutations, and 13/18 (72.2%) also had *PIK3CA* mutations. Thus, there is a significant association between *ARID1A* and *PTEN/PIK3CA* mutations.

Microsatellite instability (MSI)

MSI is a feature of the endometrioid subtype; therefore we determined the MSI status of 241/276 low-grade EECs and 13/30 EEC-3s. We found that 97/241 (40.2%) of the low-grade EECs were MSI-positive, compared with 8/13 (61.5%) EEC-3s (Supplementary Table 1).

Discussion

Endometrial carcinoma is a heterogeneous disease comprised of multiple subtypes with differing risk factors, precursor lesions, and outcomes. Lack of reproducibility in the histopathological diagnosis of endometrial carcinoma subtypes has hindered progress. For example, while some studies have found that EEC-3 and ESC have different outcomes [39], other studies have not [10]. This difference may reflect the inclusion of different cases, based on subtly different diagnostic criteria, within these cohorts. Robust and reproducible diagnostic categories are an important first step in moving towards subtype-specific treatment, as is happening for ovarian carcinoma [40,41]. However, in the case of endometrial carcinoma, it is likely that molecular markers will be needed to improve the sub-optimal performance of conventional histopathological assessment [42]. With the advent of next-generation sequencing technologies, the molecular profiles of many tumour cell types are being extensively characterized. The knowledge of these mutation profiles can potentially be used diagnostically for subclassification and to identify relevant targets for the development/deployment of targeted therapeutics. In this study, we performed exon capture sequencing of nine genes in two large cohorts of endometrial carcinomas, revealing differing mutational landscapes for endometrial carcinoma subtypes.

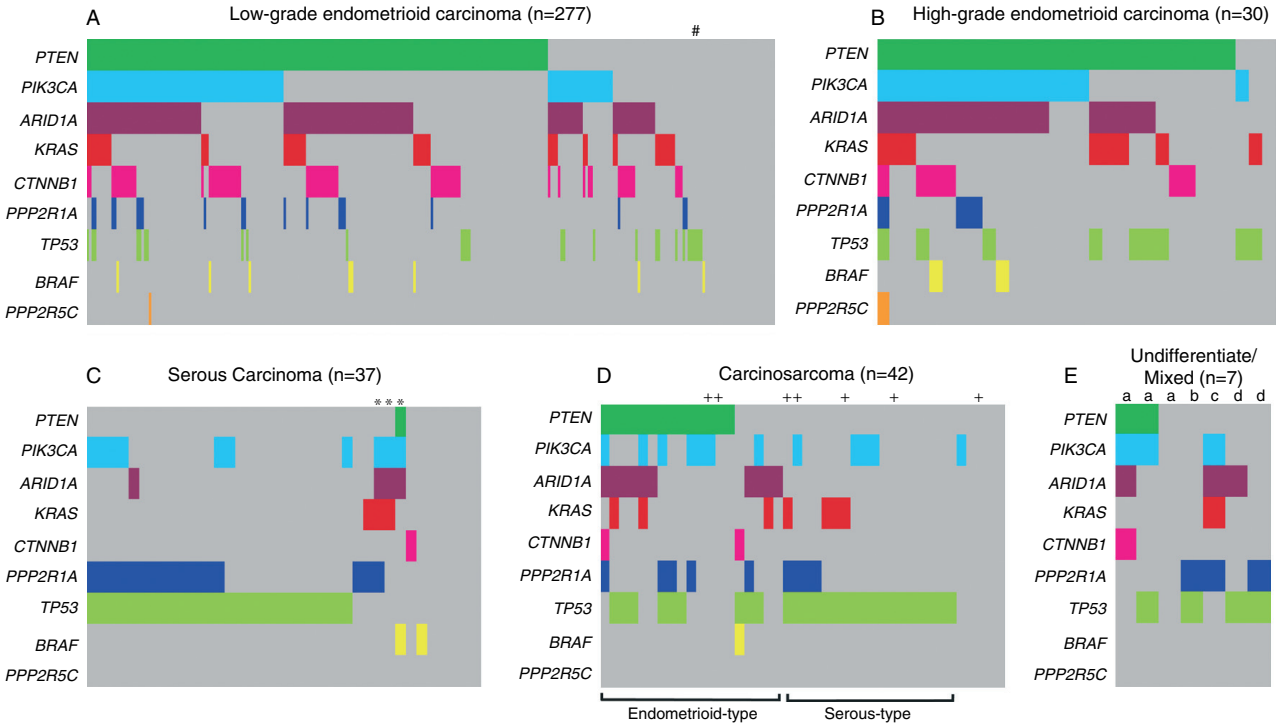

Figure 1. Mutation profiles of endometrial subtypes. (A) Low-grade endometrioid carcinoma, including grade 1 and 2 tumours ($n = 276$). (B) High-grade endometrioid carcinoma, grade 3 tumours ($n = 30$). (C) Serous carcinoma ($n = 37$). (D) Carcinosarcoma ($n = 42$); + indicates carcinosarcomas with heterologous differentiation elements. (E) Undifferentiated and mixed histology subtypes: (a) undifferentiated carcinomas; (b) mixed low-grade EEC with serous carcinoma; (c) mixed endometrioid and clear cell carcinoma; (d) mixed serous and clear cell carcinoma. Rows indicate genes; columns represent tumour cases. Coloured bars indicate mutations including missense, truncating, indels, and splice site mutations. Grey bars indicate that no mutations were detected. *Serous carcinoma outliers with *ARID1A* mutations; #low-grade EEC mutation outliers with serous-type mutations (*TP53* or *PPP2R1A*).

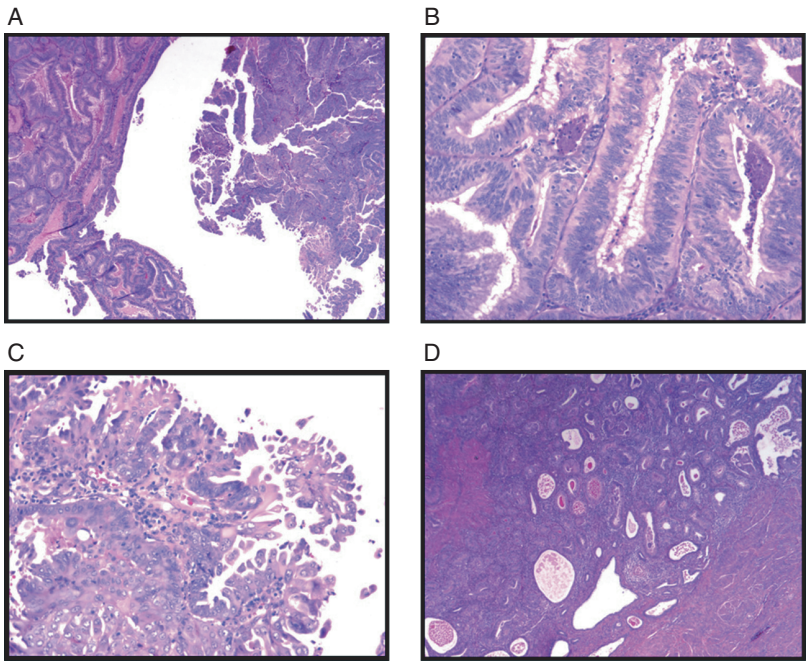

Figure 2. A case originally diagnosed as serous carcinoma, but with an *ARID1A* mutation and no *TP53* mutation, is a mixed low-grade endometrioid and serous carcinoma (case 1120). (A) A mix of a grade 1 endometrioid (left half) and high-grade serous (right half) carcinoma, 40× total magnification (original). (B) Total magnification (200×, original) image of histologically distinct low-grade endometrioid carcinoma. (C) Total magnification (200×, original) image of serous carcinoma component. Other pathologists may interpret this morphology as endometrioid, reflecting inter-observer differences in the diagnosis of these problematic cell types. The sample of tumour used for sequencing was from an area of this serous-type morphology. (D) Atypical complex hyperplasia in the background endometrium, 40× total magnification (original).

Table 5. Outlier cases with pathological review, IHC, and mutation profile

ID	841	1120	220	895	511	1034	611
Original subtype	Serous carcinoma	Serous carcinoma	Serous carcinoma	Low-grade endometrioid carcinoma	Low-grade endometrioid carcinoma	Low-grade endometrioid carcinoma	Low-grade endometrioid carcinoma
Review	Mixed serous (80%) and low-grade endometrioid carcinoma, with adjacent endometrium showing focal complex atypical hyperplasia	Mixed serous (60%) and low-grade endometrioid carcinoma, with adjacent endometrium showing complex atypical hyperplasia	Grade 3 endometrioid with clear cell changes	Grade 2 endometrioid (extensively myometrial-invasive and LVI)	Grade 3 endometrioid	Mixed low-grade (G2) endometrioid and serous carcinoma	Serous carcinoma
p53-IHC*	1	1	1	2	2	2	2
ER-IHC†	0	1	0	1	1	1	1
P16-IHC†	0	0	0	0	0	1	1
PR-IHC†	0	1	0	1	1	1	NA
PTEN-IHC‡	1	1	0	0	0	0	0
ARID1A	p.Q420* , p.R1335*	p.Q2176fs	p.Q548fs, p.G1847fs				
PTEN			p.L265fs			Splice site acceptor	
PIK3CA	p.G106V, p.V344M	p.Q546K, p.H1047Y	Y1021C				
KRAS	p.G13D	p.G12A					
PPP2R1A	p.R182W				p.P179L		
TP53				p.R282W	p.H193L	p.R248Q	p.S241F
CTNNB1							
BRAF			p.A526V, p.P403fs				
PPP2R5C							

*Scoring: 0 = loss of expression; 1 = normal expression; 2 = overexpression. †Scoring: 0 = no expression; 1 = overexpression. ‡Scoring: 1 = normal expression; 0 = loss of expression.

As demonstrated in previous studies, we identified high frequencies of mutations within *PTEN, PIK3CA, ARID1A, KRAS*, and *CTNNB1*, and lack of *TP53* mutations in low-grade EECs. EEC-3s demonstrate a similar pattern of mutations, but with a significantly increased frequency of *TP53* mutations. High frequencies of *PTEN* mutations in EECs confirm that this is an early driver event in tumour progression. Our results show that the frequency of MSI cases is similar in low-grade EEC and EEC-3, which supports the view that the majority of EEC-3s have progressed from low-grade EEC [10].

Recent studies identified a high frequency of concurrent *PTEN* and *PIK3CA* mutations in endometrial carcinomas [15,24], but not in any other tumour type investigated to date [24]. In this study, we also observed this phenomenon in low-grade EECs and EEC-3s, but not in ESC or carcinosarcoma. We determined that in low-grade EECs and EEC-3s, *ARID1A* mutations are significantly associated with concurrent mutations in *PTEN* and *PIK3CA*, a novel finding suggesting a cooperative role of these pathways in EEC tumourigenesis.

ESCs have frequent *TP53* and *PPP2R1A* mutations and lack mutations in *PTEN, ARID1A*, and *CTNNB1*, a mutational profile distinct from that of EECs. While it was not possible to classify tumours solely based on this nine-gene mutation panel, we were able to use the mutation profile as a diagnostic adjunct for morphological subclassification in individual cases. This is an attractive prospect, given the significant problems in distinguishing EEC-3 and ESC highlighted in recent studies [5,13,14,28,37,43]. We observed mutational outliers where the original diagnosis did not fit the mutation profile, specifically ESC cases with *ARID1A* mutations, and low-grade EECs with only *TP53* mutations. In most of these outlier cases, retrospective review by two independent pathologists resulted in reclassification, agreeing with the subtype-specific mutation patterns rather than the original diagnosis.

Immunostaining has been recommended as an adjunct in endometrial carcinoma subtype diagnosis [37], but has not been widely adopted. The best validated set of markers for distinguishing between EEC and ESC is ER, p53, PTEN, and p16 [5], and we applied these to 'outlier' cases in this study. A panel is recommended as none of these immunostains is completely sensitive or specific. Despite being tested in many studies, problems persist in the application of these immuno-markers and were also demonstrated in this study; specifically, there is no ability to interpret IHC profiles which are neither typically serous (PTEN- and p16-positive, ER-negative, p53 abnormal) nor endometrioid (PTEN-negative, p16-negative or weakly positive, ER-positive, p53 normal) [5,10,37,42], which was the outcome for six of seven outlier cases (Table 5). Mutational analysis potentially offers an easier-to-interpret (ie binary) output, compared with IHC, where ambiguous staining results for individual stains such as p16 are possible, but at this time it is uncertain whether mutational assessment, IHC, or a combination of both can best serve as diagnostic adjuncts in subtype diagnosis of endometrial carcinoma.

It has previously been proposed that ESC may arise through two different tumourigenic pathways, ie from progression through hyperplasia and low-grade EEC, or arising via high-grade endometrial intraepithelial carcinoma, in an oestrogen-independent pathway [44]. In this study, we observed two tumours initially diagnosed as ESC that showed an endometrioid mutation profile. On retrospective review, the diagnosis for both tumours was changed to mixed serous and endometrioid. This observation is not novel but does give further support to ESCs arising in some cases by an alternative molecular pathway, rather than the classical type 2 pathway (Figure 3 and Supplementary Figure 4) [9]. This further suggests that the classification of endometrial carcinomas cannot be encompassed by a simple dualistic model. In particular, the high-grade subtypes show considerable heterogeneity not reflected adequately in a type 1 versus type 2 model. Future studies will be required to address the following issues: (1) How reproducible is molecularly supported subtype diagnoses? (2) If diagnoses can be made reproducibly, do subtypes show significant differences in stage at diagnosis, pattern of spread, prognosis or response to treatment? Only after those questions are addressed can subtype-specific management move forward and mutation-based treatment decisions be made for challenging diagnoses.

We also investigated the molecular profiles of carcinosarcomas. These tumours are generally uncommon with a poor prognosis [45] and are composed of a mixture of carcinomatous and sarcomatous elements [46]. While previous studies have not identified a high number of mutations in this subtype [47], we showed a moderate frequency of mutations in the majority of genes sequenced. This discrepancy may be due to limited exon sequencing in previous studies; in the present study, all exons of these genes were examined. Two patterns of mutations were observed: an endometrioid-type mutation profile (*ARID1A, PTEN, PIK3CA, KRAS*) and a serous mutation profile (*TP53, PPP2R1A*). This suggests a dualistic molecular evolution of carcinosarcomas with an endometrioid-like or a serous-like mutation pattern (Figure 3). Further validation studies will be necessary to determine whether these molecular profiles are associated with different morphological features in the carcinomatous or sarcomatous components or whether they are associated with outcome differences.

We acknowledge that there are limitations of this study; we were unable to perform full histopathological reviews of many cases, including all carcinosarcomas. There were also limited numbers of cases of EEC-3 and ESC in this study; therefore independent validation studies, linked with outcome [48], will be needed in these tumour types. There is also uncertainty about

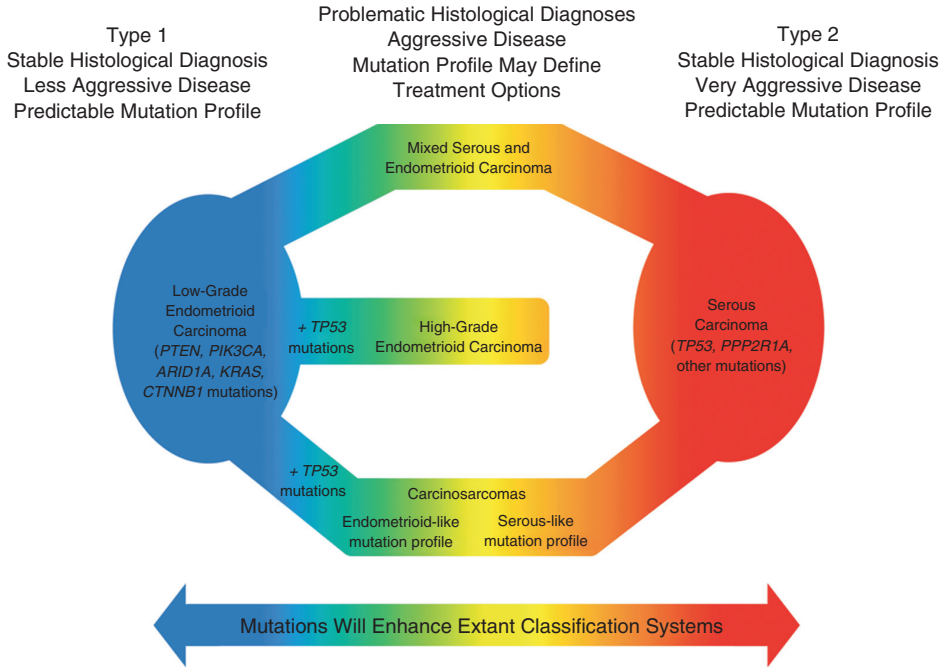

Figure 3. Mutational analysis may be an effective tool to classify morphologically problematic cases into biologically relevant treatment groups. High-grade cell types tend to be diagnostically challenging cases, often with multiple morphological features of endometrioid and/or serous carcinomas. The addition of mutation profiles can lead to reproducible diagnosis and the future of mutation-based treatment decisions for targeted therapeutics. Blue and red colours indicate distinct mutation profiles for low-grade EEC and serous carcinomas. Yellow indicates the cases where the mutational profiles will aid in separating out the appropriate histological subtype and guide appropriate treatment options for patients.

the sensitivity of the exon capture method, and false negatives are likely to be present in this data set. The TCGA endometrial sequencing effort will prove to be useful in validating the observations of this study.

In conclusion, we have identified distinct molecular profiles that may aid in endometrial carcinoma classification leading to more reproducible diagnoses. Although endometrial carcinoma subtype diagnoses and grade are currently used in guiding patient management, mutational analysis is emerging as a realistic option in clinical practice. In the future, we predict that the mutational classification of endometrial carcinomas will become an important tool in diagnosis, guiding mutation-based targeted treatment decisions. Mutation profiles are already being applied in other cancers for selecting targeted therapeutics; for example, BRAF inhibitors in malignant melanoma [49] and BRAF and EGFR targeting in colorectal cancers [50,51]. Determination of the role of mutational analysis in the assessment of endometrial carcinomas will require additional study, with careful comparison of molecular versus conventional subclassification.

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Abbreviations

EEC	endometrial endometrioid carcinoma
EEC-3	high-grade (grade 3) endometrioid carcinoma
ESC	endometrial serous carcinoma
MSI	microsatellite instability
TCGA	the cancer genome atlas
TMA	tissue microarray

Author contribution statement

MKM, SPS, MH, BG, and DGH conceived and designed the study. MKM, JD, JS, WY, MH, KT, TZ, and HM carried out experiments. BG and CHL performed pathological reviews. MM, JD, MCUC, KW, JS, WY, AT, LP, APS, DGM, JNM, SPS, PJG, BG,

CHL, and DGH collected data and performed data analysis and assisted in interpretation of data. MCUC, MKM, and JD performed statistical analysis. MKM, JD, BG, CHL, and DGH wrote the manuscript and created the figures. DGH and PJG provided endometrial samples for sequencing. All authors reviewed and approved the manuscript.

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Note: References 52–56 are cited in the Supporting information to this article.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods

Detailed materials and methods for DNA isolation, bioinformatics analysis, DNA validation, primer list, TMA and immunohistochemistry, MSI assay, and statistical and cluster analysis

Table S1. All mutation data for endometrial subtypes. Table includes cases with subtype, grade, MSI status, and all mutation data including amino acid change and genomic location (hg18).

Table S2. Mutations and IHC data of cases included on TMA. All Vancouver endometrial cases included on the TMA with IHC scores and IHC data.

Figure S1. A histogram of the probability distribution of the predicted SNV positions.

Figure S2. Box plots of the mean coverage of each gene in the cases with and without mutations.

Figure S3. Endometrial unsupervised hierarchical mutation clustering analysis aids in visualizing mutational outliers.

Figure S4. An intermediate type of high-grade endometrial carcinoma is not encompassed in the type 1 and type 2 model.