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Molecular analysis of mixed endometrial carcinomas shows clonality in most cases

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Abstract

Mixed endometrial carcinoma refers to a tumor that is comprised of two or more distinct histotypes. We studied 18 mixed-type endometrial carcinomas - 11 mixed serous and low-grade endometrioid carcinomas (SC/EC), 5 mixed clear cell and low-grade endometrioid carcinomas (CCC/EC), and 2 mixed clear cell and serous carcinoma (CCC/SC), using targeted next generation sequencing and immunohistochemistry to compare the molecular profiles of the different histotypes present in each case. In 16 of 18 cases there was molecular evidence that both components shared a clonal origin. Eight cases (6 EC/SC, 1 EC/CCC and 1 SC/CCC) showed a serous carcinoma molecular profile that was the same in both components. Five cases (3 CCC/EC and 2 SC/EC) showed a shared endometrioid molecular profile and identical mismatch repair protein (MMR) deficiency in both components. A single SC/EC case harbored the same *POLE* exonuclease domain mutation in both components. One SC/CCC and one EC/CCC case showed both shared and unique molecular features in the two histotype components, suggesting early molecular divergence from a common clonal origin. In two cases, there were no shared molecular features and these appear to be biologically unrelated synchronous tumors. Overall, these results show that the different histologic components in mixed endometrial carcinomas typically share the same molecular aberrations. Mixed endometrial carcinomas most commonly occur through morphological mimicry, whereby tumors with serous-type molecular profile show morphological features of endometrioid or clear cell carcinoma, or through underlying deficiency in DNA

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nucleotide repair, with resulting rapid accrual of mutations and intratumoral phenotypic heterogeneity. Less commonly, mixed endometrial carcinomas are the result of early molecular divergence from a common progenitor clone or are synchronous biologically unrelated tumors (collision tumors).

Keywords

Endometrial cancer; mixed carcinoma; serous carcinoma; endometrioid carcinoma clear cell carcinoma; next generation sequencing

Introduction

Mixed endometrial carcinomas contain two or more distinct histotypes of endometrial carcinoma, with at least one histotype being a type II endometrial carcinoma.¹ Each component histotype by definition has to represent more than 5% of the tumor, based on the finding that the presence of as little as 5% of a more aggressive histotype can be associated with more aggressive clinical behavior.² While the frequency of mixed carcinoma diagnosis likely varies between institutions depending on the diagnostic threshold used, mixed carcinomas can constitute 5% or more of endometrial cancer diagnoses.³⁻⁵ Excluding mixed endometrioid and undifferentiated carcinoma (also referred to as dedifferentiated carcinoma), the most common scenario for mixed endometrial carcinoma based on the literature is mixed endometrioid and serous carcinoma, followed by mixed endometrioid and clear cell carcinoma.²⁻⁶

The diagnosis of mixed carcinoma as currently defined relies predominantly on histologic features as the distinctive components present should display classic or well-accepted variant morphologic appearance of the respective histotypes. Immunohistochemistry may be used to support the presence of mixed components and a combination of p53 and p16 may be used to differentiate between endometrioid carcinoma and serous carcinoma, as most serous carcinomas show aberrant p53 immunostaining (diffuse nuclear staining or completeness absence of nuclear staining of the tumor cells) and diffuse p16 immunostaining.⁷⁻¹⁰ To distinguish between endometrioid carcinoma and clear cell carcinoma, markers such as HNF-1 β , napsin A and estrogen receptor may be useful given that great majority of clear cell carcinomas show a HNF-1 β and napsin-A-positive immunoprofile, though the same immunoprofile can be seen in a subset of serous carcinoma as well.¹¹⁻¹⁵

In recent decades, there is increasing appreciation of the extent of morphologic overlap between the different histotypes of endometrial carcinoma^{16, 17}. For instance, some endometrioid carcinomas can focally display clear cell changes that mimic the solid or papillary patterns of clear cell carcinoma,¹⁸ and some serous carcinoma may display a predominantly glandular morphology that mimics endometrioid carcinoma.¹⁹ This realization raises a fundamental question about the oncogenesis of mixed carcinoma. More specifically, do the different histotypes in mixed carcinoma arise through completely unrelated oncogenic mechanisms (collision tumor) or do they share a common oncogenic origin – i.e. either progression from one histotype to another histotype, divergence from a

common progenitor into different histotypes, or a single tumor histotype that focally displays a variant morphology that mimics a different histotype.

To gain further biologic insights into mixed endometrial carcinomas, we performed a comprehensive mutation screen and immunohistochemical analyses on 18 mixed endometrial carcinomas with spatially distinct histotype components. Our results show that the different histotype components in most mixed tumors are clonally-related and share the same molecular alterations.

Methods

Study samples

This study included 18 mixed endometrial carcinomas that were identified from the pathology archives at Vancouver General Hospital (Vancouver, Canada) and Calgary Laboratory Services (Calgary, Canada). All the cases were reviewed by two study pathologists (MK and CHL) and fulfilled the following inclusion criteria. Firstly, all mixed endometrial carcinomas were from hysterectomy specimens and contained two different histological types of endometrial carcinoma, with at least one being a type II carcinoma, as defined by WHO 2014.¹ Secondly, the different histotypes displayed classic or well-recognized variant histologic features and formed spatially distinct areas that are not intimately admixed, such that the tissue from the different histotype components can be cored separately (0.6 mm tissue cores). The diagnoses in these cases were made based on morphologic findings only. We excluded cases that were difficult to histotype because of the presence of ambiguous histologic features throughout the tumor (without spatially distinct areas of different histological histotypes).²⁰ The study was approved by the Institutional Review Board.

Immunohistochemistry and interpretation

All immunohistochemistry analysis was performed on representative whole tissue sections from hysterectomy specimens. For HNF-1 β , napsin A, p53 and mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2), the primary antibodies used and the staining methods are the same as that reported previously.^{12, 14, 21, 22} The mouse monoclonal p53, clone DO-7 (catalog number: M7001) antibody obtained from Dako (Burlington, Ontario, Canada), the rabbit polyclonal HNF-1 β (catalog HPA002083) antibody obtained from Sigma (St. Louis, Missouri, USA) and the rabbit polyclonal Napsin A (catalog 352A) antibody obtained from Cell Marque (Rocklin, California, USA). For MMR proteins, slides were incubated with MLH1 (mouse monoclonal antibody, 1:50 dilution, cat#:NCL-L-MLH1, clone ID:ES05, Leica Microsystems, Newcastle, United Kingdom), MSH2 (mouse monoclonal antibody, 1:1000 dilution, cat#286M-16, clone ID:G219-1129, Cellmarque, Rocklin, CA, USA), MSH6 (rabbit monoclonal antibody, 1:200 dilution, cat#:CLAC-0047, clone ID:EP49, Cedarlane Corporation, Burlington, ON, Canada) and PMS2 (rabbit monoclonal antibody, 1:20 dilution, cat#:CLAC-0049, clone ID:EP51, Cedarlane Corporation, Burlington, ON, Canada) and processed using the Leica Bond Max platform (Leica Microsystems, Wetzlar, Germany) as per manufacturer's protocol with proprietary reagents. The detection system used was the Bond polymer refine.

Napsin A cytoplasmic staining was quantified based on percentage tumor cell staining and tumors showing $\geq 5\%$ staining was considered positive. HNF-1 β immunostain was considered to be positive if the tumor exhibited at least moderate nuclear staining intensity in $> 70\%$ of tumor cells.¹² P53 immunostain was considered to be aberrant (mutated/inactivated) if the tumor exhibited 1) diffuse moderate to strong uniform nuclear staining in $\geq 70\%$ of the tumor cells (diffuse), or 2) complete absence of nuclear staining in the tumor cells in the presence of focal nuclear staining of the stromal cells (complete absent). Immunostain for p53 was considered normal (wild type pattern) if any degree of non-diffuse nuclear staining ($<70\%$) of the tumor cells was present.²³ For MLH1, MSH2, MSH6 and PMS2, staining was considered abnormal/absent when there is loss of nuclear expression by the tumor cells compared to internal positive control (stromal fibroblasts and inflammatory cells).

DNA extraction

For each case, tissue cores (0.6 mm diameter) of different histological components and of the corresponding normal tissues were obtained from formalin fixed paraffin embedded (FFPE) blocks. The tumor cores were derived from the most superficial aspect of the tumor (adjacent to the endometrial cavity) and they contained only histologically viable tumor with minimal inflammatory infiltrates. To ensure that each tissue core contained only the histological component that it was supposed to represent, individual tumor FFPE block was flipped over and re-embedded to produce a H&E slide from the opposite side of the block, and tissue cores that were contaminated by a different histological type were excluded from further analysis. Normal tissue that was distant from and uninvolved by endometrial carcinoma was used for comparison, and the examples of the corresponding normal tissue examined included cervix, fallopian tube and ovary. DNA was extracted from the tissue cores using the Qiagen FFPE DNA extraction kit based on manufacturer's protocols.

Targeted gene panel sequencing analysis and validations

We performed sequencing analysis to detect mutations in 26 genes that have been previously found to be recurrently mutated in endometrial carcinomas.²⁴⁻²⁹ These included ABCC9, ARID1A, ARID5B, CCND1, CHD4, CSMD3, CTCF, CTNNB1, EP300, FBXW7, GRLF1, FGFR2, KRAS, MAP3K4, PIK3CA, PIK3R1, PIK3R2, POLE, PPP2R1A, PTEN, RBMX, RPL22, SPOP, TP53, TSPYL2 and *ZFHX3*, similar to that described previously.¹⁴ The Illumina custom TruSeq amplicon panel was designed using Illumina's DesignStudio and included 1440 amplicons (175bp) that covers 98% of the exons and untranslated regions (UTR) of these 26 genes. Custom amplicon libraries were prepared starting with 250ng of FFPE DNA as per Illumina's Custom TruSeq Library Preparation protocol. Before pooling, normalization was performed by quantifying individual libraries using the Qubit fluorometer, then pooled based on equal concentrations. Library pools were then quantitated for amplifiable libraries using the Kapa Biosystems FAST qPCR SYBR quantification kit based on manufacturer's protocols. Pooled TruSeq libraries were sequenced using the Illumina MiSeq using 300 cycle V2 kits. Analysis was performed using the Miseq Reporter and somatic variant caller 3.2.3.0, then filtered against normal germline reads to determine somatic status. Only somatic non-synonymous mutations passing quality filter with at least

10% variant allele frequency were further evaluated. These mutations were manually checked in bam files using Integrated Genome Viewer, and validated orthogonally by direct Sanger sequencing using primer sets that target the regions containing the mutations. Mutations that were common between the different histologic components were further confirmed to be somatic in nature by analyzing DNA derived from corresponding normal tissue.

Results

Clinical and pathological features of the mixed endometrial carcinoma

Eighteen mixed endometrial carcinomas were studied, and these included 11 mixed EC and SC, 5 mixed EC and CCC, and 2 mixed SC and CCC tumors. The clinical and pathologic features of these 18 cases are summarized in Table 1. All patients were post-menopausal in status. The mean age was 69 years for the patients with mixed EC and SC, 69 year for patients with mixed EC and CCC and 67 years for patients with mixed SC and CCC. Clinical follow-up was available for 16 of the 18 patients (Table 1) and 7 patients had limited follow-up with a follow-up period of less than 2 years.

For mixed carcinomas with EC and SC, the proportion of SC component ranged from 35 % to 90 % of the tumors (average of 70%). All cases showed direct contact between the two components except one (case 11) where the apparent EC and SC components involved different parts of an endometrial polyp. The EC component demonstrated prototypical endometrioid histology with predominantly glandular or villoglandular architecture and all were low-grade (FIGO grade 1 or grade 2) tumors that displayed low-grade nuclear features (lacking nuclear pleomorphism) (Figure 1). None of the EC components exhibited definitive squamous differentiation though three showed squamoid areas and one showed mucinous differentiation. Mitotic rates in the EC components were variable between cases, ranging from 1 to 25/10 high power fields (HPF) (1.5 mm²). The SC component demonstrated prototypical serous histology with predominantly papillary architecture, prominent papillary/luminal surface tumor cell budding and cellular detachment (Figure 1). There was high-grade nuclear atypia present in all cases and three cases showed prominent nuclear pleomorphism (> 3:1 variation in nuclear size) with tumor giant cells. Mitotic figures were readily identified, ranging from 14 to 48 /10 HPF.

For mixed carcinomas with EC and CCC, the proportion of CCC component ranged from 10 % to 85 % (average of 47%). The EC components were low-grade (FIGO grade 1 or 2) in all cases and demonstrated typical endometrioid histologic features (Figure 2A-D). Squamous differentiation was present in the EC component of one tumor. Mitotic rates in the EC component ranged from 2 to 10 /10 HPF. The CCC component displayed exclusively papillary architecture in 1 case, exclusively solid architecture in 1 case and a mix of architectural patterns (papillary/glandular/solid) in 3 cases (Figure 2A-D). Hobnail cells were present in all cases that contain papillary/glandular areas. The nuclear features in the CCC component were of higher grade (grade 2-3) compared to the corresponding EC component, with tumor giant cells present in the CCC component in one case (case 16). There was at least moderate amount of cytoplasm present in all cases, with the cytoplasm being predominantly clear in 4 cases and eosinophilic in 1 case. Hyalinized stroma was

present in the cores of the papillae or around the glands in all cases. Mitotic rates ranged from 4 to 15/10 HPF.

For mixed carcinomas with SC and CCC, the SC component in case 17 predominantly involved the surface and the superficial glands of an endometrial polyp while the CCC involved the same endometrial polyp as well as the background endometrium. The SC component in this case showed a mix of glandular and papillary architecture with high grade nuclear features and high mitotic rate (20 MF/10 HPF) (Figure 2E-F). In contrast, the CCC component exhibited a mix of solid and papillary architectures with hobnail cells and cytoplasmic clearing. Stromal hyalinization was prominent and the mitotic rate (6 MF/10 HPF) was lower than the corresponding SC component. In case 18, the SC component showed papillary architecture with high-grade nuclei and surface tumor budding (Figure 2H). The CCC component showed a mix of tubulocystic and glandular architecture with hobnail cells that featured clear to eosinophilic cytoplasm (Figure 2G). Mitotic rates were comparable between the SC (15 MF/10 HPF) and CCC (13 MF/10 HPF) components.

Immunohistochemical and genetic features of mixed endometrioid and serous carcinoma

We performed targeted whole-exon sequencing of selected genes that have been shown to be recurrently mutated in endometrial cancers (including endometrioid, serous and clear cell histotypes), using DNA extracted from on the different histologic components of mixed endometrial carcinoma. The coding regions of 26 endometrial cancer genes (except for exon 1 of *ARID1A* which was not covered because of primer design constraint) were sequenced using a custom-designed amplicon-based targeted sequencing panel. Of the 36 tumor samples (18 pairs), there was an average of 821-fold coverage per amplicon (range 430 to 2070-fold) and 94% of the amplicons had a median coverage in the 36 tumor samples of at least 50-fold. We also performed p53 and MMR protein immunohistochemistry on these cases and scored the staining results of the different histologic components separately. Of the 11 mixed carcinomas with SC and EC, we identified somatic mutations in 9 cases (Table 2). The mutations were either identical or partially shared between the SC and EC components in 7 of 9 cases. Of the 7 cases with identical or partially shared mutations, 3 MMR-intact tumors (case 2, 3 and 7) harbored prototypical serous-type mutations with concurrent somatic *TP53* and *PPP2R1A* (hotspot) mutations with notable absence of *PTEN*, *ARID1A*, *CTNNB1* or *KRAS* mutations in both the histologically apparent EC and SC components, while one MMR-intact tumor (case 1) showed the same missense somatic *TP53* mutations in both components. This was further confirmed by p53 immunohistochemistry that demonstrated aberrant p53 staining in the corresponding EC and SC components in these cases (Figure 3A-D). Two other tumors (case 4 and 8) showed the same somatic mutations in *ARID1A*, *PTEN* and *RPL22* between the corresponding EC and SC components. Both tumors showed wild-type p53 staining and were MMR-deficient with the same pattern of MMR protein loss in the corresponding EC and SC components (case 4 and 8). The remaining case (case 9) with partially shared mutations between the EC and SC components harbored *POLE* exonuclease domain mutation (L424V) and showed isolated MSH6 loss in both components (Figure 1E-F and 3E-F). This tumor possessed a large number of point mutations, with some mutations being shared by both EC and SC components and other

mutations that were unique to either the EC or SC component only (Table 2). Immunohistochemistry for p53 showed wild-type staining in both EC and SC components.

Among the 9 mixed EC and SC with demonstrable mutations, two cases (case 10 and 11) showed completely different mutations between the corresponding EC and SC components. The EC component in both cases harbored prototypical endometrioid-type mutations (*PTEN* and *ARID1A* mutations) while the SC component harbored prototypical serous-type mutations (*TP53*, *PPP2R1A* and/or *FBXW7*). The corresponding EC and SC components also displayed discordant p53 immunostaining patterns with aberrant diffuse nuclear staining in the SC component and wild-type staining in the EC component (Figure 1G-H and 3G-H). Case 10 showed intact MMR protein expression in both components, while case 11 showed intact MMR protein expression in the SC component but concurrent MLH1 and PMS2 loss in the EC component. Case 11 was the only mixed EC and SC case where the components were not direct contact with each other.

There were two mixed EC and SC cases (case 5 and 6) in which no mutations were demonstrated in either the EC or SC component. These tumors showed intact MMR protein expression and the same aberrant p53 staining pattern (diffuse staining in case 5 and complete loss of staining in case 6) in the corresponding EC and SC components. The similar alteration in p53 and the concordant lack of demonstrable mutations observed in the corresponding EC and SC component suggests molecular commonality.

Immunohistochemical and genetic features of mixed endometrioid and clear cell carcinoma

Among the 5 mixed endometrial carcinomas with EC and CCC components, 4 were MMR-deficient tumors in which the same pattern of MMR protein loss was found in the corresponding EC and CCC components (Figure 4). All 4 tumors showed wild-type p53 expression, and the CCC components were all positive for HNF-1 β and napsin A, while the EC components were all negative for these markers (Figure 4A-B). Genetically, the EC and CCC components in 3 of the 4 MMR-deficient tumors showed shared somatic mutations involving genes such as *PTEN*, *ARID1A* and *RPL22*, indicating a common genetic origin (Table 3). In case 15, both the EC and the CCC components showed the same pattern of MMR protein deficiency (isolated MSH6 loss). While there were several mutations identified in both components, there were no mutations that were in common between the apparent EC and CCC components. The remaining mixed EC and CCC tumor (case 16) showed identical *TP53* frameshift mutation and a loss of tumoral p53 expression in both the endometrioid component (napsin A/HNF-1 β -negative) and the clear cell component (napsin A/HNF-1 β -positive). There were no additional molecular abnormalities demonstrated in either component. We interpreted this as a serous-type endometrial carcinoma with endometrioid-like morphology and clear cell-like morphology in different areas of the tumor.

Immunohistochemical and genetic features of mixed serous and clear cell carcinoma

There were 2 mixed endometrial carcinomas with SC and CCC in our series, and both showed intact MMR protein expression (Figure 3). The CCC component in case 17 was

positive for HNF-1 β and napsin A while the SC component was negative for these markers (Figure 5A-B). Our mutation screen showed two somatic point mutations in *PIK3CA*, with N345I (C2 domain of *PIK3CA*) present in both the SC and CCC components and M1004I (exon 20 *PIK3CA* mutation) found only in the SC component. Furthermore, the SC component showed aberrant p53 staining while the CCC component showed wild-type p53 staining (Figure 5C-D). Histologically, the SC component was focally confined to an endometrial polyp while the CCC component involved the same endometrial polyp as well as the background endometrium. There was no direct contact between the 2 components in the histologic sections sampled. Despite the shared genetic commonality (*PIK3CA* N345I), the observed immunoprofile suggests early divergence in the tumorigenic development that resulted in synchronous serous carcinoma and clear cell carcinoma. In case 18, the CCC component was positive for napsin A and negative for HNF-1 β while the SC component was negative for both markers (Figure 5E-F). The SC and CCC components harbored identical somatic *TP53*, *PPP2R1A* and *PIK3CA* mutations, and both showed aberrant (strong nuclear) p53 staining (Figure 5G-H). These molecular findings suggest that the histologically apparent SC and CCC components in this case represent a single tumor type.

Discussion

Mixed endometrial carcinomas constitute a small subset of endometrial carcinomas, but they present a significant diagnostic challenge. In this study, we molecularly characterized the individual histotype components in 18 mixed endometrial carcinomas and found that the histologically distinct components in most of the mixed endometrial carcinomas shared the same molecular alterations. The Cancer Genome Atlas (TCGA) studies recently proposed a molecular classification scheme for endometrial carcinomas based comprehensive genetic, epigenetic and proteomic analyses of a large series of cases.²⁴ This proposed molecular classification approach has been further validated with respect to its clinical significance.³⁰⁻³² The recognition of the different molecular subtypes of endometrial carcinomas gives us a general framework to interpret our findings here. Using the molecular classification scheme proposed by TCGA and mismatch repair protein (MMR) immunohistochemistry as a surrogate marker for high microsatellite instability (MSI-H),^{24, 33} our results showed that mixed endometrial carcinomas can be separated into four different molecular categories – 1) Serous molecular type with areas resembling other histotype (i.e. endometrioid carcinoma or clear cell carcinoma), 2) Hyper- or ultramutated endometrioid molecular type characterized by nucleotide repair deficiency (with *POLE* exonuclease domain mutation or MMR-deficiency), 3) Tumors showing molecular divergence from common progenitor, and 4) Collision tumors showing no molecular commonalities. Categories 3 and 4 are considered as true mixed tumors in this discussion.

Serous molecular type with areas resembling other endometrial histotype

Based on TCGA finding, the serous/serous-like molecular type of endometrial carcinoma harbored frequent *TP53* mutations with *PPP2R1A* and/or *FBXW7* mutations occurring in a smaller subset, and lacked evidence of a high degree of microsatellite instability (MSI-H) and *POLE* exonuclease domain mutations. There were 8 cases (case 1, 2, 3, 5, 6, 7, 16 and 18) that showed identical serous-type molecular abnormalities between the corresponding

histologic components (concurrent *TP53* and *PPP2R1A* mutations, or *TP53* abnormality only without other demonstrable molecular abnormalities). These included 6 histologically mixed EC/SCs, 1 histologically mixed EC/CCC, and 1 histologically mixed SC/CCC. In the mixed EC/SC scenario, it is increasingly recognized that a subset of serous carcinoma can exhibit tubuloglandular architecture that histologically mimics endometrioid carcinoma.^{19, 20} Similar to tubo-ovarian high-grade serous carcinoma, endometrial serous carcinomas also harbor *TP53* mutations and show a high degree of somatic copy number alterations,²⁴ which reflect chromosomal instability. However, these somatic copy number alterations may not be homogeneous across the tumor as demonstrated by intratumoral heterogeneity of DNA ploidy, a surrogate for copy number alterations.³⁴ Consequently, nuclear grade may also vary.

Cases 16 (EC/CCC) and case 18 (SC/CCC) exhibited identical serous-type molecular profile in both components with compelling morphological and immunohistochemical evidence for the clear cell component (Napsin A-positive) and the endometrioid or serous component (HNF-1 β /napsin A-negative). We have previously observed that a subset of *TP53*-mutated pure endometrial clear cell carcinomas harbor prototypical serous-type mutation profiles (with concurrent *TP53* and *PPP2R1A* mutations, in the absence of *ARID1A* or *PTEN* mutations).¹⁴

These 8 mixed-type cases are all serous molecular type endometrial carcinomas that morphologically mimic other histotypes (low-grade endometrioid carcinoma or clear cell carcinoma). Our findings are in line with the TCGA data whereby the majority of mixed tumors clustered in the copy number high cluster 4 and are serous-like with respect to their molecular type.²⁴ Immunohistochemical analysis of p53 expression can aid in the recognition in these cases of morphologic mimicry. If p53 immunostaining results suggest *TP53* mutation (diffuse strong tumor nuclear staining or a complete absence of tumor nuclear staining in the presence of appropriate internal stromal positive control) in both apparent histologic components, the most likely diagnosis is serous carcinoma (with endometrioid-like area and/or clear cell-like area), as these tumors show serous-like molecular profiles. However, we do acknowledge that accurate classification of tumors with pure clear cell morphology, abnormal *TP53* and expression of napsin A/ HNF-1 β requires further study and ideally a greater insight into the defining molecular features of endometrial clear cell carcinoma.

Hypermutated/ultramutated molecular type with MMR-deficiency/*POLE* mutation

There were 5 tumors (cases 4, 8, 12, 13, and 14) that exhibited an identical pattern of MMR-deficiency and 1 tumor (case 9) that harbored identical *POLE* exonuclease domain mutation between the corresponding histologic components. The mutation profiles in these cases were endometrioid in type. Case 9 was a *POLE* ultramutated endometrial carcinoma (case 9) with a *POLE* exonuclease domain mutation (L424V) and a loss of MSH6 expression that was present in both the endometrioid and the serous components. This *POLE* exonuclease domain mutation was previously documented to be present in endometrial carcinoma with

an ultramutated genomic landscape and a subset of *POLE*-mutated tumors can also be MSI-H.²⁴ A large number of somatic mutations (point mutations and small insertions/ deletions) were identified in this case, some of which were common but some were different between the corresponding endometrioid and serous component. The 5 MMR-deficient tumors as a group also harbored a high number of somatic mutations,²⁴ and two (cases 4 and 8) showed mixed endometrioid and serous histologic components. In these 3 mixed EC/SC cases with DNA nucleotide repair deficiency (MMR-deficiency and/or *POLE* exonuclease domain mutation), the serous-like component exhibited prototypical histologic features of serous carcinoma, with at least focally high-grade nuclear features, significant mitotic activity (> 10 MF/10 HPF), prominent nuclear stratification/tumor budding and papillary architectural features. In contrast, the corresponding endometrioid component showed typical endometrioid features with villoglandular or glandular architecture, smooth luminal border and low nuclear grade. While it is unclear whether the presence of serous-like area in these molecular contexts portends any clinical significance, it is worth noting that the serous-like area in our 3 cases lacked evidence of *TP53* mutation (a disease-defining event in serous carcinoma) by sequencing and immunohistochemistry. Therefore, it is more likely that the presence of such serous-like areas reflects the tendency for *POLE*-ultramutated endometrioid carcinoma and MMR-deficient endometrioid carcinoma to display serous-like features, as observed previously.³⁵ It is important to note that *POLE*-ultramutated tumors have an excellent prognosis when managed by conventional treatment algorithms and that MMR-deficient (MSI-H) tumors have a better prognosis than serous-type endometrial carcinoma.^{24, 31, 32} We therefore believe based on current evidence that it is important not to mistaken these tumors as serous carcinoma or mixed carcinoma with serous component because of their better prognosis compared to typical *TP53*-mutated pure serous carcinoma.⁶

Three (cases 12, 13, and 14) of the four mixed EC/CCCs were MMR-deficient and displayed the same pattern of MMR-deficiency between the corresponding endometrioid and clear cell components. It is important to note that the clear cell components in cases 12, 13 and 14 all showed prototypical clear cell immunoprofile (napsin A and HNF-1 β -positive) while the corresponding endometrioid components were all immuno-negative for napsin A and HNF-1 β .^{12, 15} Overall, our findings here show a high frequency of MMR deficiency in mixed EC and CCC tumors (75%). Given that MMR deficiency is rare in pure endometrial CCC,^{14, 36, 37} it appear likely these MMR-deficient mixed EC and CCC tumors are biologically different from pure endometrial CCC. It is however unclear whether these MMR-deficient (MSI-H) tumors with mixed endometrioid and clear cell morphology would behave clinically more like MMR-deficient (MSI-H) tumors with pure endometrioid histology or MMR-intact pure endometrial CCC, particularly with in terms of therapeutic response. Future studies are needed to address these questions.

Tumor showing early molecular divergence from common progenitor

One of the 2 mixed SC/CCC cases (case 17) showed intact MMR expression and wild-type *POLE* exonuclease domain in both the clear cell component (napsin A/HNF-1 β -positive) and the serous component (napsin A/HNF-1 β -negative). The serous component also harbored an exon 20 *PIK3CA* mutation (M1004I) that was not identified in the clear cell component. While these findings would suggest that these are two unrelated and distinct

tumors – synchronous serous carcinoma and clear cell carcinoma, they do share in common a somatic activating mutation involving the C2 domain of *PIK3CA*.^{38, 39} Histologically, both the serous component and the clear cell component involved an endometrial polyp (with serous component being confined to the polyp), with no direct contact between the two components. It is plausible that the serous and the clear cell components share the same precursor progenitor cells in the endometrial polyp that harbored this *PIK3CA* (N345I) mutation, with the serous component going on to acquire a further mutation in exon 20 of *PIK3CA* and alterations involving *TP53*, and with the clear cell component presumably going on to acquire other molecular abnormalities. We therefore interpret this case as an example of early molecular divergence from a common progenitor clone.

In case 15 (mixed EC/CCC), there were several mutations identified in each histologic component, but none were shared by both components. Both components demonstrated the same pattern of MMR deficiency (MSH6 loss only), and both were negative for napsin A and HNF-1 β by immunohistochemistry. Based on these findings, the most probable explanation is that there is likely a germline MSH6 mutation (Lynch syndrome) that led to the development of two synchronous MSH6-deficient endometrioid carcinomas (one showing endometrioid morphology and one showing clear cell morphology). This is however speculative in nature as we do not know whether this patient has Lynch syndrome. More importantly, it is also unclear whether MMR-deficient (MSI-H) pure clear cell carcinoma behaves similarly as MMR-intact pure endometrial clear cell carcinoma in the endometrium. Therefore, one may also consider placing case 15 in category 2 (hypermutated molecular type) if the presence of either focal or diffuse clear cell features in a MMR-deficient setting is clinically and therapeutically different from typical MMR-intact clear cell carcinomas.

Collision tumors

There were 2 cases in our series (cases 10 and 11) that showed different molecular alterations in the different histologic components with no overlap at all, and both were mixed EC/SC in histology. These appear to represent biologically unrelated tumors that have occurred synchronously in the endometrium (collision tumor). The endometrioid component in these two cases harbored endometrioid-type mutations (*PTEN* and/or *ARID1A*) and displayed wild-type p53 immunostaining pattern, while the corresponding serous component harbored serous-type mutations (*TP53* and/or *PPP2R1A*) and displayed mutated p53 immunostaining pattern. Furthermore, the endometrioid carcinoma component in case 11 was MMR-deficient while the corresponding serous carcinoma component showed intact MMR protein expression. These findings demonstrate that the corresponding endometrioid and serous components in these two cases are molecularly distinct. It is important to note that we did not identify any cases in our series that may have progressed from low-grade endometrioid to serous carcinoma. Coenegrachts *et al* recently studied a series of mixed endometrial EC/SC and found that the individual histologic components shared the same mutations in 30% of the cases but displayed different mutations in 35% of cases.⁴⁰ This is in keeping with our findings that some of the mixed EC/SC shared molecular commonalities (i.e. serous carcinomas with areas that resemble endometrioid carcinoma or hypermutated/

ultramutated molecular subtype) while some mixed EC/SC appear to represent true collision tumors.

Proposed diagnostic approach to suspected mixed endometrial carcinoma

With the molecular insights gained here, we would like to recommend the use of p53 and MMR immunohistochemistry and *POLE* exonuclease domain sequencing (by Sanger or next generation sequencing) on all suspected cases of mixed endometrial carcinoma. If there is identical MMR deficiency and/or *POLE* exonuclease mutation present in the different histologic components, the case should be diagnosed as an endometrioid carcinoma with MMR-deficiency and/or *POLE* exonuclease domain mutation, and be further qualified that it exhibits mixed serous or clear cell features (to facilitate communication to pathology colleagues). If MMR deficiency and/or *POLE* exonuclease domain mutation is only observed in one of the histologic components, this would provide support for a mixed-type carcinoma (either as true collision tumor or as molecular divergence from common progenitor clone). One would subsequently rely on p53 immunohistochemistry to determine what the remaining histologic component may be. For instance, case 11 showed MMR-deficiency and wild-type p53 immunostaining in the histologically endometrioid component and intact MMR proteins but mutated p53 immunostaining in the histologically serous component. It was therefore a synchronous MMR-deficient endometrioid carcinoma and MMR-intact serous carcinoma (collision tumor). If the different histologic components both show intact MMR expression and wild-type *POLE* exonuclease domain, one would need to evaluate p53 immunostaining in each of the histologic components. Based on our findings here, it is likely that a majority of these cases will turn out to display the same mutated p53 staining pattern in the different histologic components, indicating that these are serous-type endometrial carcinomas with areas that mimic other endometrial histotypes. While this proposed diagnostic approach utilizes immunomarkers (p53 and MMR proteins) that are available in most pathology laboratories, sequencing for *POLE* exonuclease domain mutation is not available in most laboratories at the present. We however anticipate that it will become more widely available as an ancillary molecular test, given the strong prognostic significance of *POLE* exonuclease domain mutation in endometrial carcinoma, particularly among tumors with high-grade histologic features^{24, 31, 32, 41}.

In summary, our studies on mixed endometrial carcinomas show that most mixed endometrial carcinomas diagnosed based on morphologic features represent a single tumor type that demonstrates varied histologic appearances (morphologic mimicry/intratumoral phenotypic heterogeneity). Tumors with DNA repair defects, with resulting intratumoral heterogeneity, are the second largest group. We however also confirmed that true mixed endometrial carcinomas that are composed of genetically distinct synchronously-occurring endometrial carcinoma histotypes do occur, albeit rarely. These results suggest that the integration of selected ancillary studies such as p53 and MMR protein immunohistochemistry, and mutation analysis of *POLE* exonuclease domain should be considered when dealing with a mixed endometrial carcinoma, as these results may provide useful diagnostic insights.

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Figure 1. Representative H&E images of mixed endometrioid and serous carcinomas A-B). The endometrioid component (A) and the serous component (B) of case 1. C-D). The endometrioid component (C) and the serous component (D) of case 3, E-F). The endometrioid component (E) and the serous component (F) of case 9, G-H). The endometrioid component (G) and the serous component (H) of case 11.

Figure 2. Representative H&E images of mixed endometrioid and clear cell carcinomas, and mixed serous and clear cell carcinomas

A-B). The clear cell component (A) and the endometrioid component (B) of case 12, C-D). The clear cell component (C) and the endometrioid component (D) of case 13, E-F). The clear cell component (E) and the serous component (F) of case 17, G-H). The clear cell component (G) and the serous component (H) of case 18.

Figure 3. p53 and MMR protein immunostaining in mixed endometrioid and serous carcinomas
 In case 1, the endometrioid component (A) and serous component (B) of case 1 both demonstrated diffuse strong nuclear p53 immunostaining, in keeping with the presence of the same missense *TP53* mutation in both components. In case 2, the endometrioid component (C) and serous component (D) both demonstrated a complete absence of nuclear p53 immunostaining, in keeping with the presence of the same frameshift *TP53* mutation in both components. In case 9, the endometrioid component (E) and serous component (F) both demonstrated a loss of MSH6 expression immunohistochemically. In case 11, the endometrioid component (G) demonstrated wild-type p53 immunostaining while the serous component (H) demonstrates diffuse strong p53 immunostaining, in keeping with the finding of a missense *TP53* mutation in the serous component but not in the endometrioid component.

Figure 4. Immunostaining results in mixed endometrioid and clear cell carcinomas

In case 12, the clear cell component showed positive napsin A immunostaining (A) while the endometrioid component lacked napsin A immunostaining (B). Both the clear cell component (C) and the endometrioid component (D) showed a loss of MLH1 expression immunohistochemically. In case 13, both the clear cell component (E) and the endometrioid component (F) showed a loss of MSH6 expression immunohistochemically. Both the clear cell component (G) and the endometrioid component (H) showed a wild-type p53 immunostaining.

Figure 5. Immunostaining results in mixed serous and clear cell carcinomas

In case 17, the clear cell component showed positive napsin A immunostaining (A) while the serous component lacked napsin A immunostaining (B). The clear cell component showed wild-type p53 immunostaining (C) while the serous component shows diffuse strong nuclear p53 immunostaining of the tumor cells (D). In case 18, the clear cell component showed positive napsin A immunostaining (E) while the serous component lacked napsin A immunostaining (F). Both the clear cell component (G) and the serous component (H) showed diffuse strong nuclear p53 immunostaining of the tumor cells.

Table 1

Clinicopathologic features of the 18 patients with mixed endometrial carcinomas.

Case	Age	Diagnosis	% component	FIGO stage	Treatment	Follow-up
1	77	G1 EC and SC	65% and 35%	1A	TAH-BSO, adjuvant chemo-radiation	0.5 year, NED
2	64	G1 EC and SC	35% and 65%	1A	TAH-BSO	4.2 years, NED
3	74	G2 EC and SC	20% and 80%	3C2	TAH-BSO, adjuvant radiation	3.8 years, AWD
4	62	G1 EC and SC	15% and 85%	1A	TAH-NSO	5.8 years, NED
5	87	G1 EC and SC	20% and 80%	1A	TAH-BSO	0.5 year, NED
6	67	G2 EC and SC	30% and 70%	3A	TAH-BSO, adjuvant radiation	4.8 years, NED
7	80	G1 EC and SC	10% and 90%	3B	TAH-BSO (refused adjuvant therapy)	1.7 years, AWD
8	60	G1 EC and SC	50% and 50%	2A	TAB-BSO, adjuvant chemotherapy	5.1 years, NED
9	51	G1 EC and SC	50% and 50%	1A	TAH-BSO, adjuvant chemotherapy	2 years, NED
10	72	G1 EC and SC	10% and 90%	3A	TAH-BSO (refused adjuvant therapy)	Lost to follow-up
11	66	G1 EC and SC	20% and 80%	1A	TAH-BSO	1.8 years, NED
12	77	G2 EC and CCC	30% and 70%	1B	TAH-BSO	Lost to follow-up
13	61	G1 EC and CCC	40% and 60%	1A	TAH-BSO	1.9 years, NED
14	71	G2 EC and CCC	90% and 10%	1B	TAH-BSO	2.5 years, NED
15	53	G1 EC and CCC	15% and 85%	1A	TAH-BSO	6.5 years, NED
16	82	G2 EC and CCC	90% and 10%	3C2	TAH-BSO (refused adjuvant therapy)	0.2 year, DOD
17	75	SC and CCC	15% and 85%	1A	TAH-BSO, adjuvant radiation	4 years, NED
18	58	SC and CCC	20% and 80%	1A	TAH-BSO, adjuvant chemo-radiation	1 year, NED

G1: FIGO grade 1; G2: grade FIGO grade 2; EC: endometrioid carcinoma; SC: serous carcinoma; CCC: clear cell carcinoma; TAH-BSO: total hysterectomy and bilateral salpingo-oophorectomy; NED: no evidence of disease; AWD: alive with (recurrent) disease; DOD: died of the disease.

Table 2

Immunohistochemical and genetic findings in the different components of mixed endometrioid and serous carcinomas.

Case	IHC	Mutations			IHC
		Not shared	Shared	Not shared	
	Endometrioid component		Serous component		
1	p53 (mutated/diffuse), MMR (intact)		TP53 (Y102C)		p53 (mutated/diffuse), MMR (intact)
2	p53 (mutated/absent), MMR (intact)		PIK3R1 (f.s. x 2), TP53 (f.s.), PPP2R1A (P179R), CHD4 (E138D)		p53 (mutated/absent), MMR (intact)
3	p53 (mutated/diffuse), MMR (intact)		PPP2R1A (R183Q), TP53 (R117S)		p53 (mutated/diffuse), MMR (intact)
4	p53 (wild-type), MMR (MLH & PMS2 loss)		ARID1A (f.s.), RPL22 (f.s.), CCND1 (P287S), CSMD3 (T3180K)		p53 (wild-type), MMR (MLH1 & PMS2 loss)
5	p53 (mutated/diffuse), MMR (intact)				p53 (mutated/diffuse), MMR (intact)
6	p53 (mutated/absent), MMR (intact)				p53 (mutated/absent), MMR (intact)
7	p53 (mutated/diffuse), MMR (intact)		PPP2R1A (S256F), FBXW7 (R399Q), TP53 (V84M)		p53 (mutated/diffuse), MMR (intact)
8	p53 (wild-type), MMR (MSH2 & MSH6 loss)	PIK3CA (C420R), SPOP (R198W), EP300 (T594M)	ARID1A (V1982I), ARID1A (f.s.), PTEN (Q298X), PIK3CA (MIL), ARID5B (M332T)	PIK3CA (V344M)	p53 (wild-type), MMR (intact)
9	p53 (wild-type), MMR (MSH6 loss)	CSMD3 (S609Y), CHD4, ZFHX3 (R1183H, E1900K), EP300 (I1612V)	POLE (L424V, R1879C), ARID1A (G2087R), FBXW7 (R385C), PIK3R1 (A277T), MAP3K4 (E154K, R558H), PTEN (R173H), PPP2R1A (R183W, R527C), CDH4 (V395I), FGFR2 (A67V, C62Y), ZFHX3 (G3507S, K1209N), GRLF1 (F376L, P1334L), CSMD3 (A3525T, P2054S, G927D, C794Y), EP300 (M187L, R1599H)	CTNNB1 (F99L), MAP3K4 (A1202V, G1458D), ZFHX3 (E3263D, L861F), PPP2R1A (H497Y), EP300 (R705Q, A2354T)	p53 (wild-type), MMR (MSH6 loss)
10	p53 (wild-type), MMR (intact)	PIK3CA (E542K), PTEN (D92E), ARID1A (R1989X and f.s.), RPL22 (f.s.), CSMD3 (I2936R, R2561Q), CTCF (f.s.)		PPP2R1A (R183Q)	p53 (mutated/diffuse), MMR (intact)
11	p53 (wild-type), MMR (deficient; MLH1 and PMS2 loss)	PIK3R1 (f.s.), PTEN (L181R, R233X), PTEN (deletion), GRLF1 (S670X), RPL22 (f.s.)		TP53 (A138S), FBXW7 (Y465C), CSMD3 (P976R)	p53 (mutated/diffuse), MMR (intact)

IHC: immunohistochemistry; MMR: mismatch repair protein; f.s.: frame-shift mutation; HNF1B: HNF-1p.

Table 3

Immunohistochemical and genetic findings in the different components of mixed endometrioid and clear cell carcinomas, and mixed serous and clear cell carcinomas.

Case	IHC	Mutations			IHC
		Not shared	Shared	Not shared	
	Endometrioid component			Clear cell component	
12	p53 (wild-type), MMR (MLH1 & MSH2 loss), HNF1B (negative), Napsin A (negative)	MAP3K4 (N1401T), CSMD3 (G3565C, P2687S, L1254I), PTEN (R173C, D326G), CHD4 (R1183C), ZFHX3 (G3517D), FGFR2 (R190W)	CSMD3 (R1276C),SPOP (R121Q), PPP2RIA (R527H), ARID1A (f.s.), PTEN (f.s.), RPL22 (f.s.) and TPSYL2 (f.s.)	ARID1A (R2233W), RPL22 (G51R), CTNNB1 (A230T), PIK3R1 (L637M), CSMD3 (T1930I, S1448Y, A1235T, K410E, L49F), EP300 (R353C, R2185Q), ZFHX3 (A2263V)	p53 (wild-type), MMR (MLH1 & MSH2 loss), HNF1B (positive), Napsin A (positive)
13	p53 (wild-type), MMR (MSH2 & MSH6 loss), HNF1B (negative), Napsin A (negative)		PTEN (R173C, f.s.), ARID1A (Q499X, f.s.), RPL22(f.s.), CTCF (f.s.), PIK3CA (R88Q)	ARID1A (A1272V), PTEN (T286I), PIK3CA (deletion), GRLF1 (f.s.), ZFHX3 (L1539I)	p53 (wild-type), MMR (MSH2 & MSH6 loss), HNF1B (positive), Napsin A (positive)
14	p53 (wild-type), MMR (MLH1 & PMS2 loss), HNF1B (negative), Napsin A (negative)		ARID1A (H688P, f.s.), PTEN (G129A, C296X), RPL22 (f.s.), ARID5B (T663I), GRLF1 (D1008N), PIK3CA (E542K), ZFHX3 (f.s.)	ARID5B (f.s.)	p53 (wild-type), MMR (MLH1 & PMS2 loss), HNF1B (positive), Napsin A (positive)
15	p53 (wild-type), MMR (MSH6 loss), HNF1B (negative), Napsin A (negative)	PIK3CA (F83S, F542G, C604R), FBXW7 (R385C), CHD4 (A1028T), TSPYL2 (G141D)		FGFR2 (V452I), CHD4 (S1815F), CTCF (E112K, A171T), ZFHX3 (T3700A, A3009T, N2210T, P2095L, A623T), GRLF1 (G827R), PPP2RIA (R183W, G396D), EP300 (V279A, R568W, R838C, M1157I), RPL22 (f.s.)	p53 (wild-type), MMR (MSH6 loss), HNF1B (negative), Napsin A (negative)
16	p53 (mutated/loss), MMR (intact), HNF1B (negative), Napsin A (negative)		TP53 (f.s.)		p53 (mutated/loss), MMR (intact), HNF1B (positive), Napsin A (positive)
	Serous component			Clear cell component	
17	p53 (mutated/diffuse), MMR (intact), HNF1B (negative), Napsin A (negative)	PIK3CA (M1004I)	PIK3CA (N345I)		p53 (wild-type), MMR (intact), HNF1B (positive), Napsin A (positive)
18	p53 (mutated/diffuse), MMR (intact), HNF1B (negative), Napsin A (negative)		TP53 (G105C), PPP2RIA (P179R), PIK3CA (G106V), CHD4 (E138D)		p53 (mutated/diffuse), MMR (intact), HNF1B (negative), Napsin A (positive)

IHC: immunohistochemistry; MMR: mismatch repair protein; f.s.: frame-shift mutation; HNF1B: HNF-1p.