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POLE exonuclease domain mutation predicts long progression-free survival in grade 3 endometrioid carcinoma of the endometrium

Bo Meng [a], Lien N. Hoang [b], John B. McIntyre [c], Máire A. Duggan [d], Gregg S. Nelson [e], Cheng-Han Lee [a,*,1], Martin Köbel [d,1]

[a] Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada
[b] Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada
[c] Department of Oncology, University of Calgary and Translational Laboratory, Tom Baker Cancer Centre, Calgary, AB, Canada
[d] Department of Pathology and Laboratory Medicine, University of Calgary and Calgary Laboratory Services, Calgary, AB, Canada
[e] Department of Gynecologic Oncology, Tom Baker Cancer Centre and University of Calgary, Calgary, AB, Canada

HIGHLIGHTS

- Newly identified *POLE* exonuclease domain mutations occur in about 15% of grade 3 endometrioid carcinoma of the endometrium.
- *POLE* exonuclease domain mutation in grade 3 endometrial endometrioid carcinoma is associated with very favorable outcome.
- *POLE* exonuclease domain mutation is an important genetic biomarker that can be used to guide clinical management.

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ABSTRACT

Objective. *POLE* exonuclease domain mutations were recently found to occur in a subset of endometrial carcinomas and result in defective proof-reading function during DNA replication. The aim of this study is to further characterize the clinical and pathologic significance of *POLE* exonuclease domain mutations in high-grade endometrial carcinomas.

Methods. We assessed for mutations in the exonuclease domain of *POLE* by Sanger sequencing in 53 grade 3 endometrioid, 25 serous, 16 clear cell and 5 dedifferentiated carcinomas. We correlated *POLE* mutation status with clinicopathologic features and molecular parameters. Univariate and multivariate survival analyses were performed using Kaplan–Meier and cox regression analyses.

Results. *POLE* exonuclease domain mutations were identified in 8 of 53 (15%) grade 3 endometrioid carcinomas and not in any other histotypes examined. Only 1 of the 8 grade 3 endometrioid carcinomas with *POLE* exonuclease domain mutation displayed deficient mismatch repair protein expression by immunohistochemistry (MSH6 loss), compared to 21 of 45 grade 3 endometrioid carcinomas with wild-type exonuclease domain. When analyzed together with published grade 3 endometrioid carcinomas by The Cancer Genome Atlas, the presence of *POLE* exonuclease domain mutation was associated with significantly better progression-free survival in univariate ($p = 0.025$) and multivariate ($p = 0.010$) analyses, such that none of the patients with *POLE* mutated tumors experienced disease progression

Conclusions. *POLE* exonuclease domain mutations occur in a subset of grade 3 endometrioid carcinomas and are associated with good clinical outcome. It can serve as an important prognostic molecular marker to guide the management of patients with grade 3 endometrioid carcinomas.

Introduction

DNA polymerases such as POLE are responsible for DNA synthesis and replication in human cells [1]. They contain proof-reading exonuclease domain that functions to ensure low mutation rates in replicating cells [1–3]. Missense mutations of the exonuclease domain of *POLE* have recently been documented in melanoma, lung carcinoma, colorectal carcinoma and endometrial carcinoma [4–9], and are associated with

* Corresponding author at: Department of Laboratory Medicine and Pathology, University of Alberta and Royal Alexandra Hospital, Room DTC 5002-16, 10240 Kingsway, Edmonton, AB T5H 3V9, Canada. Fax: +1 780 735 4715.
E-mail address: chlee2@ualberta.ca (C.-H. Lee).
[1] Both authors contributed equally to this study.

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a high number of genetic mutations across the tumor genome [3–5]. In endometrial carcinomas, two studies reported *POLE* exonuclease domain mutations in 8% of 167 and 248 tumors, respectively [4,5]. In both cohorts, *POLE* exonuclease domain mutations were more common in high-grade (grade 3) endometrial carcinomas than low-grade (grade 1 or 2) endometrial carcinomas. With respect to histologic tumor types, *POLE* exonuclease domain mutation is rare in serous carcinoma based on The Cancer Genome Atlas (TCGA) Research Network dataset, as it was identified in only 1 of 48 tumors (2%), while such mutation was found in 2 of 8 serous carcinomas (25%) by Church et al. [4]. In three other separate studies that focused on endometrial serous carcinomas, Zhao et al. and Le Gallo et al. found *POLE* exonuclease domain mutation in 4 of 57 tumors (7%) and 1 of 13 tumors (8%) respectively [10,11], while Kuhn et al. did not identify *POLE* mutations in any of the 10 tumors analyzed [12]. With respect to clinical outcome, endometrial carcinomas with *POLE* exonuclease domain mutation exhibit a less aggressive clinical course as compared to endometrial carcinomas without *POLE* exonuclease domain mutation based on TCGA data [5].

These prior studies suggest a potential prognostic role for *POLE* exonuclease domain mutations in high-grade endometrial carcinoma, as it may help to identify a subset of endometrial carcinoma with high-grade histology but relatively indolent clinical course. However, further validation is needed. In addition, it remains unclear whether *POLE* exonuclease domain mutations correlate with tumor histologic type (i.e. endometrioid, serous and clear cell type), as there was no mention of centralized pathology review in some of the prior studies. In this study, we attempt to provide further insights into the clinical and pathologic significance of *POLE* exonuclease domain mutation in a series of rigorously reviewed high-grade endometrial carcinomas.

Materials and methods

Study cohorts

This study used two cohorts: Our local series referred to as high-grade endometrial carcinoma (HGEC) cohort and The Cancer Genome Atlas (TCGA) data. The HGEC cohort consists of 99 high-grade endometrial carcinomas diagnosed and treated at the Tom Baker Cancer Centre, Calgary, AB, Canada, between 2005 and 2011. All of the cases were evaluated histologically, with a consensus established based on the histology slides review by at least 4 gynecologic oncology subspecialty pathologists, and immunohistochemically, using a seven marker panel. The tumor types assigned here represent a combination of consensus diagnoses with immunohistochemical marker information [13,14]. Clinical data was obtained by retrospective chart abstraction. Disease specific death was defined as death from disease excluding death from other causes, and progressive-free survival represents time to clinical/radiologic evidence of disease progression from initial diagnosis. Ethical approval was received from institutional research ethics boards. For TCGA cohort, we downloaded clinical data and mutational data for 16 selected genes via the webapi interface of the cBIO portal (http://www.cbioportal.org/public-portal/) using the query:

> "webservice.do?cmd=getClinicalData&cancer_study_id=ucec_tcga_pub&case_set_id=ucec_tcga_pub_all" on Jan 24, 2014 [15,16]. This included 373 cases and 250 had annotated mutations, from which we identified 49 grade 3 endometrioid carcinomas and 44 serous carcinomas.

DNA extraction

DNA was extracted from 99 formalin fixed paraffin embedded (FFPE) tumor samples from hysterectomy specimens as previously described [17]. All patient tumor blocks underwent pathology review to determine histology and to clearly identify tumor regions with high (>80%) tumor cellularity. DNA purity and yield were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

POLE exonuclease domain polymerase chain reaction (PCR)

Primer sets that cover the exonuclease domain regions of *POLE* in which mutations were previously identified in endometrial carcinomas were used to amplify exonuclease domain genomic regions — exon 9 (forward: 5′-TGTTCAGGGAGGCCTAATGG-3′; reverse: 5′-AACAAATACTAACAGTGGGG-3′), exon 10 (forward: 5′-GCTGCAATTCTGATCTGACG-3′; reverse: 5′-CAGCCTCTGACTTGTGCTGA-3′), exon 11 (forward: 5′-CTTCTGAACTTTGGGAGAGG-3′; reverse: 5′-CACCTCCTAAGTCGACATGG-3′), exon 12 (forward: 5′-GCATTAGAGCCTGACCTGC-3′; reverse: 5′-ACAGCACAGTCTGCAAGAGG-3′), exon 13 (forward: 5′-CGGGATGTGGCTTACGTGC-3′; reverse: 5′-TTGCATCTGTCTGTGTGGTG-3′), exon 14 (forward: 5′-TCTGTGCTTCACACTTGACC-3′; reverse: 5′-GACATCCACCTCCATTCAGC-3′). PCR amplifications were performed as previously described using 50 ng genomic DNA and the primer sets using High-Fidelity Tag DNA polymerase (Invitrogen, Carlsbad, CA, USA) [18]. Prior to sequencing, PCR amplicons were electrophoresed and visualized to confirm the presence of desired amplicons and the absence of off-target amplification products. PCR amplicons were then purified using Axygen™ AxyPrep Mag™ PCR Clean-up Kits according to manufacturer's protocol (Axygen Biosciences, Union City, CA, USA) and resuspended in 30 μl double-distilled water.

Direct Sanger sequencing

Direct bi-directional sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA) with 96 capillaries. Sequencing reactions were performed as previously described and all mutation positive samples were re-sequenced to confirm mutational status [17].

Statistical analysis

POLE exonuclease domain mutation status across the different tumor types was assessed with contingency analysis and statistical differences were quantified using the two-sided Fisher's exact test for ordinal data and ANOVA test of variance for continuous data. Univariate progression-free, disease-specific, and overall survival analyses were evaluated by generating Kaplan–Meier curves and log-rank statistics were applied. Multivariate progression-free and overall survival analyses were evaluated by cox regression models adjusted for age as continuous variable and FIGO stage (stage I/II versus III/IV). For all analyses, a p-value < 0.05 was considered statistically significant. Statistical analyses were performed on JMP v 10.0.0 (SAS Institute, Cary, NC, U.S.A.).

Results

Clinical and pathologic features

Our HGEC cohorts included 99 high-grade endometrial carcinomas and these encompassed 53 grade 3 endometrial endometrioid carcinoma (EC3), 25 endometrial serous carcinomas (ESC), 16 endometrial clear cell carcinomas (CCC) and 5 endometrial dedifferentiated carcinomas. The clinical features of EC3 and ESC are summarized and compared with the TCGA data in Table 1. The average age for patients with EC3 was 62 years, which was significantly younger than patients with ESC (average age of 69 years) ($p = 0.007$) and CCC (average age of 73 years) ($p < 0.001$). The mean age of the dedifferentiated carcinoma patients was 66 years. A slight majority (56%) of patients with SC presented with stage 3–4 disease, while 35% of patients with EC3, 19% of

Table 1
Clinical and pathologic features of study cohorts.

	G3 endometrioid carcinoma			Serous carcinoma		
	HGEC	TCGA [5]	p-Value	HGEC	TCGA	p-Value
N	53	49		25	43	
Age (mean, years)	62	61	0.80	69	70	0.80
Stage III/IV	20/53 (38%)	15/48 (31%)	0.53	14/25 (56%)	25/44 (59%)	0.81
Recurrence	16/53 (30%)	11/47 (23%)	0.50	14/23 (61%)	16/41 (39%)	0.12
Mean follow-up of censored cases for recurrence (months)	51	41	0.13	42	37	0.58

G3, grade 3; HGEC, refers to our high-grade endometrial carcinoma cohort; TCGA, The Cancer Genome Atlas data [5]. p-Value refers to differences between EC and ESC using the ANOVA test of variance for continuous data and two-sided Fisher's exact test for ordinal data.

patients with CCC and 75% of patients with dedifferentiated carcinoma presented with stage 3–4 disease.

The pathologic and molecular significance of POLE exonuclease domain mutation in high-grade endometrial carcinomas

Among the 99 high-grade endometrial carcinomas, 8 tumors harbored missense mutations in the exonuclease domain of *POLE*. Six of the 8 mutations were previously described and shown to be somatic, 5 in exon 9 (P286R and S297F) and 1 in exon 13 (V411L). Two novel exon 9 mutations (833C>T) were identified; both were shown to be somatic in nature. This novel exon 9 mutation results in a missense mutation (T278M) within the exonuclease domain of POLE (a.a 268–471). In terms of tumor type, all 8 tumors harboring *POLE* exonuclease domain mutations were EC3 and represented 15% of the EC3 in the present series (Table 2). None of the 25 SC, 16 CCC and 5 dedifferentiated carcinomas harbored *POLE exonuclease domain* mutations. This distribution is similar to the TCGA data, which showed 8 cases with *POLE* mutations within the exonuclease domain (Table 2).

Of the 8 *POLE*-mutated grade 3 endometrioid carcinomas, none harbored *PIK3CA* hotspot (exon 9 or 20) mutation, in contrast to 10 of 45 tumors with wild-type *POLE* exonuclease domain that showed *PIK3CA* hotspot mutations ($p = 0.072$) [17]. In terms of the immunohistochemical expression of mismatch repair proteins (MLH1, PMS2, MSH2 and MSH6) [19], only 1 of the 8 tumors showed loss of mismatch repair protein(s) (in the form of isolated MSH6 loss), in contrast to 21 of 45 tumors with wild-type *POLE* exonuclease domain that showed loss of mismatch repair protein(s) ($p = 0.054$).

The clinical significance of POLE exonuclease domain mutation in grade 3 endometrioid carcinoma (EC3)

We combined the EC3 patients from the HGEC cohort ($n = 53$) with TCGA cohort ($n = 49$) for the following analyses, as the two EC3 cohorts showed similar characteristics in terms of patient age and disease stage (Table 1). The clinical features of the patients with *POLE* (exonuclease domain)-mutated EC3 and the patients with *POLE* wild-type EC3 are shown in Table 3. There were no significant differences between these two groups of patients in terms of age, body mass index and the proportion with FIGO stage 1 disease. Treatment information was available only for the HGEC cohort and there was no significant difference in the proportion of patients that received adjuvant radiation therapy in between the groups. In the HGEC cohort, none of the patients with *POLE* (exonuclease domain)-mutated EC3 received adjuvant chemotherapy, in contrast to about half with *POLE* wild-type EC3 that received adjuvant chemotherapy (52%, $p = 0064$). This appears due to a predominance of low stage disease (FIGO stage 1, in 7 of 8 patients) in the *POLE*-mutated tumors of the HGEC cohort, though 6 of the 7 FIGO stage 1 EC3 showed inner half myometrial invasion, and 3 of 7 demonstrated lymphovascular invasion.

Three analyses were performed to evaluate the prognostic significance of *POLE* exonuclease domain mutations in EC3. The first analysis evaluated progression-free survival and combined the data on EC3 from the HGEC cohort ($n = 53$) with TCGA cohort ($n = 49$) (Fig. 1). The second analysis evaluated disease-specific survival in our present HGEC cohort of 53 grade 3 endometrioid carcinomas (Fig. 2) because only disease-specific survival data was not available from the TCGA. The third analysis evaluated overall survival in the combined HGEC and TCGA cohorts (Fig. 3). As shown in Fig. 1, none of the patients whose tumors harbored *POLE* exonuclease domain mutations experienced disease progression (median follow-up 33 months), while about a third of the patients with tumors showing wild-type *POLE* exonuclease domain experienced disease progression at 5 years (5-year progression-free survival 65%, standard error 6.2, median follow-up for censored cases 36 months, mean 46 months) (log rank test $p = 0.025$). The presence of *POLE* exonuclease domain mutations remained a significant prognostic parameter for progression-free survival ($p = 0.010$) in multivariate analysis adjusted for age ($p = 0.24$, as continuous variable) and FIGO stage ($p < 0.0001$, stage I/II versus III/IV). For the HGEC cohort, the disease-specific survival analysis showed a similar trend, as none of the

Table 2
Prevalence of exonuclease domain *POLE* mutation across histotypes in our HGEC study cohort and TCGA data.

Histotypes	HGEC	TCGA [5]	p-value
G3 endometrioid	8/53 (15%)	8/49 (16%)	1.00
Serous	0/25	1/43 (2%)	1.00
Clear cell	0/16	NA	
Dedifferentiated	0/5	NA	
Total	8/99 (8%)	9/93 (10%)	1.00

G3, grade 3 endometrioid carcinoma; HGEC, refers to our high-grade endometrial carcinoma cohort; TCGA, The Cancer Genome Atlas data [5]. p-Value two-sided Fisher's exact test.

Table 3
Associations of clinical and pathologic parameters with *POLE* exonuclease domain mutation status in combined EC3 from the HGEC and TCGA cohorts.

	EC3 *POLE* mutated	EC3 *POLE* wild type	p-value
N	16	86	
Age (mean)	58.6	62.3	0.23
BMI	26.1	28.6	0.36
LVI	3/8 (38%)	22/45 (49%)	0.27
FIGO 2009 stage			
Stage I	11/16 (69%)	49/85 (58%)	0.46
Ia	5/7	15/22	
Ib	2/7	7/22	
Stage II	1/16	5/85	
Stage III	4/16	19/85	
Pelvic node positive	0/8	9/45	
Paraaortic node positive	0/8	4/45	
Stage IV	0/16	12/85	
Adjuvant therapy*			
Radiation	2/8 (25%)	15/44 (34%)	0.61
Chemotherapy	0/8 (0%)	23/44 (52%)	0.0064

EC3, grade 3 endometrioid carcinoma. HGEC, refers to our high-grade endometrial carcinoma cohort; TCGA, The Cancer Genome Atlas data; BMI, body mass index; LVI, lymphovascular invasion. p-Value using the ANOVA test of variance for continuous data and two-sided Fisher's exact test for ordinal data.

* Data available only for HGEC cohort.

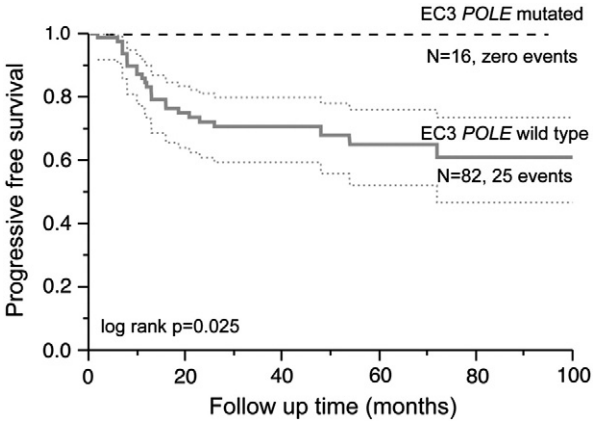

Fig. 1. Progression-free survival analysis in grade 3 endometrial endometrioid-type carcinoma stratified based on *POLE* exonuclease domain mutation status in combined HGEC and TCGA [5] cohorts. EC3, grade 3 endometrioid carcinoma. Dotted lines, 95% confidence intervals.

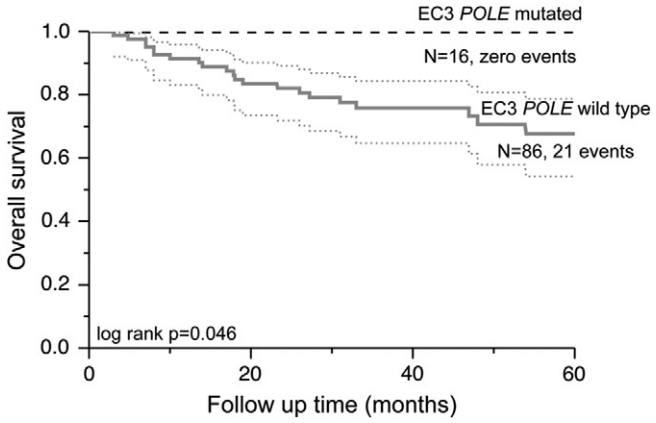

Fig. 3. Overall survival analysis in grade 3 endometrial endometrioid carcinoma stratified based on *POLE* exonuclease domain mutation status in combined HGEC and TCGA [5] cohorts. EC3, grade 3 endometrioid carcinoma. Dotted lines, 95% confidence intervals.

8 patients with *POLE* exonuclease domain mutated tumors died from their tumor, while about a third of the patients with *POLE* exonuclease domain wild-type tumors succumbed to their disease at 5 years (5-year disease specific survival 65%, standard error 8.4, median follow-up for censored cases 45 months, mean 51 months, rank test $p = 0.12$) (median follow-up 36 months). While the presence of *POLE* exonuclease domain mutations was a significant prognostic factor for overall survival in EC3 ($p = 0.046$, Fig. 3), it was not significant in multivariate analysis ($p = 0.053$) adjusting for age and disease stage.

Discussion

In our present series, we identified *POLE* exonuclease domain mutations only in EC3, while none of the 26 SC, 16 CCC and 5 dedifferentiated carcinomas harbored these mutations. This is comparable to what was reported by TCGA where only 1 of 48 (2%) SC was mutated [5]. This outlier case demonstrated a prototypical SC genotype (with concurrent *TP53* and *PPP2R1A* mutations, in the absence of *PTEN* or *ARID1A* mutations, and lacking hypermutation genetic landscape), and the histology of this tumor upon review (based on the online scanned image) was also compatible with SC. Another study reported *POLE* exonuclease domain mutation in 2 of 8 (25%) SC examined [4], while others found *POLE* exonuclease domain mutation in 4 of 57 (7%), 1 of 13 SC (8%) and 0 of 10 SC (0%) [10–12]. There was however no specific mention of centralized pathology reviews in these studies. Given the known difficulty in reliable histologic tumor subtyping of high-grade endometrial carcinomas [13,20–23], it is plausible that some of these *POLE* exonuclease domain mutated SC may have been misclassified. In support of this notion, 2 of the 4 *POLE* exonuclease domain mutated SC identified in one study were described as mixed-type tumor with serous carcinoma component, which suggests that these cases were likely difficult to subtype [11]. Moreover, the single case of index SC was found by Le Gallo et al. to harbor *POLE* exonuclease domain mutation and to exhibit a hypermutation genetic profile and lacked either *TP53* or *PPP2R1A* mutations, a finding that is not typical for SC [10]. It therefore appears that the frequency of *POLE* exonuclease domain mutation in prototypical SC is likely very low.

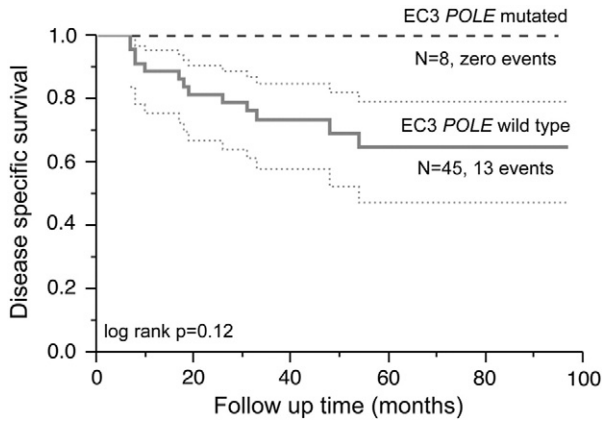

Fig. 2. Disease-specific survival analysis in grade 3 endometrial endometrioid carcinoma stratified based on *POLE* exonuclease domain mutation status in the HGEC cohort. EC3, grade 3 endometrioid carcinoma. Dotted lines, 95% confidence intervals.

For low-grade endometrioid carcinoma (grade 1 or 2), *POLE* exonuclease domain mutations have been identified in about 6% of tumors [4,5]. Among EC3, we identified *POLE* exonuclease domain mutations in 8 of 53 (15%) tumors in the present series, which is also comparable to the 16% (8 of 49) reported by TCGA and about 22% (7 of 32) reported by Church et al. [4,5]. This constitutes a significant subset of endometrioid carcinomas with high-grade histology which is typically regarded as being more aggressive clinically. Our survival analyses shown here further support the initial association made by TCGA that *POLE* exonuclease domain mutated tumors appear to behave non-aggressively with current clinical management. More specifically, by combining the cohorts of EC3 patients between TCGA and the present study series, we observed significantly better progression-free survival in both univariate and multivariate analyses for patients with tumors showing *POLE* exonuclease domain mutations compared to patients with tumor showing wild-type *POLE* exonuclease domain, as none of the *POLE* exonuclease domain mutated EC3 in either cohorts experienced disease progression during the follow-up period. As disease-specific survival data was not available for the TCGA cohort, disease-specific survival analysis of our present series showed a similar trend, though the difference was not statistically significant, which was likely due to the small number of patients in the *POLE* exonuclease domain mutated group. It is important to note also that none of the patients with *POLE* exonuclease domain mutated EC3 died from their tumors or died from any other causes within 5 years of disease diagnosis.

Missense mutation(s) involving the well-conserved exonuclease domain of *POLE* can affect the proof-reading function of the DNA polymerase, resulting in hypermutation [2,3]. In the case of endometrial carcinoma, there are two major hotspots for *POLE* mutations within the exonuclease domain based on review of Catalogue of Somatic Mutations in Cancer (COSMIC) database [24]. The most common hotspot is in amino acid residue 286 (encoded by exon 9), with 15 tumors previously reported to harbor missense mutation P286R. The second most common hotspot is in amino acid residue 411 (encoded by exon 13), with

7 tumors previously reported to harbor missense mutation V411L. In addition, there are other amino acid regions in exon 9 (amino acid 275 in one tumor and 297 in three tumors), exon 13 (amino acid 428, 444, 446 and 453 in one tumor each) and exon 14 (amino acid 456 in two tumors and 465 in one tumor) in which missense mutations have been previously documented in endometrial carcinomas. When compared to constitutional DNA, these missense mutations have been found to be somatic in type in all except one patient who harbored germline R446Q [2]. In our series, P286R was also the most common missense mutation identified. We also identified a novel somatic missense mutation — T278M, in two EC3. This missense mutation affects an amino acid (278) that is located near the exonuclease catalytic site residue 275 [2] and such a mutation would likely alter the conformation of the active site, thereby affecting its proof-reading function.

It is worth noting that we observed a low frequency of mismatch repair protein loss in *POLE* exonuclease domain mutated endometrioid carcinomas. This is in keeping with prior observations that *POLE* exonuclease domain mutations and mismatch repair protein mutations infrequently co-exist in either endometrial or colorectal carcinomas [25]. As Kim et al. had previously speculated, it is possible that microsatellite-unstable genome cannot tolerate additional mutation burden introduced by *POLE* exonuclease domain mutation, and when it co-exists with mismatch repair protein mutation(s), it may represent a later event [25].

Mechanistically, it is tempting to speculate that tumors harboring *POLE* exonuclease domain mutations mutate at such excessively high frequency that prevents purposeful and optimal tumor evolution. Heitzer and Tomlinson suggested that such ultra-mutations might be functionally suboptimal with respect to the 'classical' mutations [3]. In support of this, all eight *POLE* exonuclease domain mutated EC3 in the TCGA data showed mutation in at least one of the MMR genes, but only two of these cases were MSI-high. *POLE* exonuclease domain mutated EC3 shows several mutations in genes associated with endometrial oncogenesis but commonly outside the hotspots. For example, among the *POLE* exonuclease domain mutated EC3 in the TCGA cohort and our cohort, none of the tumors harbored activating *PIK3CA* mutations in the classic hotspots (E545 or H1047) were present in our study or the TCGA data, yet in the TCGA data 6 out of 8 cases had *PIK3CA* mutation at positions outside these hotspots.

In summary, the presence of *POLE* exonuclease domain mutation in EC3 is a strong molecular prognosticator predicting a low risk of disease progression with current clinical management. These findings advocate for the integration of *POLE* exonuclease domain mutation status in the management of patients diagnosed with grade 3 endometrioid carcinomas.

Conflict of interest statement

The authors declare no conflict of interest.

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