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Prognosis and treatment response stratification according to loss of proofreading (LOP) *POLE* variants

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ABSTRACT

Background Only a subset of polymerase epsilon (*POLE*) mutations is associated with hypermutant phenotype; we hypothesized that only loss-of-proofreading (LOP) *POLE* mutations are associated with favorable immunotherapy response.

Methods This retrospective cohort study included a pan-cancer cohort of 69,223 patients from cBioPortal and a cohort of patients with 41 *POLE* mutant metastatic colorectal (CRC) treated with immunotherapy at the MD Anderson Cancer Center between January 2017 and May 2023. We evaluated prognosis according to *POLE* mutation functionality.

Results In the pan-cancer cBioPortal cohort (n=69,223) *POLE* was mutated in 2.8% (1,965) of tumors; of these, only 7.5% (n=148) had LOP *POLE* mutations (0.0% of entire cohort). Endometrial cancer (6.6%) and CRC (1.2%) were the only tumor types with greater than 1% incidence of LOP *POLE* mutation. Overall survival was similar between non-LOP *POLE* and *POLE* wildtype patients (HR=0.94, 95% CI 0.82 to 1.07, p=0.34); conversely, LOP *POLE* patients had significantly better outcomes (HR=0.23, 95% CI 0.16 to 0.32, p<0.0001). In the clinical cohort, all nine patients with LOP *POLE* mutations achieved durable clinical benefit (objective response rate (ORR) 88.9%, complete response rate (CR) 33.3%, disease control rate (DCR) 100%) and median progression-free survival (PFS) was not reached at the time of analysis, after median follow-up of 32 months. None of the nine patients with microsatellite stable (MSS), non-LOP *POLE* tumors achieved an ORR of 0%, with median PFS of 3.7 months (HR 0.05 LOP relative to non-LOP *POLE*, 95% CI 0.01 to 0.19, p<0.0001). Interestingly, median PFS on first-line cytotoxic agents was significantly shorter for patients with LOP *POLE* compared with patients with MSS non-LOP *POLE* mutant tumors (2.1 vs 9.7 months, HR 3.33, 95% CI 0.87 to 12.74, p=0.012).

Conclusions Identifying the subset of *POLE* mutations that cause LOP is critical to distinguish patients likely to respond to immunotherapy. Patients with CRC with LOP *POLE* mutant tumors experienced deep, sustained response to immunotherapy but were resistant to standard cytotoxic chemotherapy, in stark contrast to those with non-LOP *POLE* mutations.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Polymerase epsilon (*POLE*) mutation has been proposed as a biomarker for immunotherapy response, but there are conflicting reports regarding which specific point mutations are associated with this benefit.

WHAT THIS STUDY ADDS

⇒ Metastatic patients with colorectal cancer (CRC) with loss of proofreading (LOP) *POLE* mutations demonstrated poor response to standard front-line cytotoxic chemotherapy but excellent response to immune therapy. Patients with non-LOP *POLE* mutant tumors had outcomes similar to those with *POLE* wild-type tumors.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study highlights the critical importance of stratifying *POLE* variants into LOP and non-LOP categories and suggests that immune therapy should be the front-line treatment for patients with metastatic CRC with LOP *POLE* mutant tumors.

INTRODUCTION

A subset of DNA polymerase epsilon (*POLE*) mutations cause proofreading defects while preserving polymerase function, resulting in error-prone DNA replication and remarkably high tumor mutational burden (TMB).[1–3] In these tumors, an increase in tumor-infiltrating lymphocytes (TILs) has consistently been seen relative to *POLE* wild-type tumors.[4 5] Taken together, *POLE* mutational status has been proposed as a predictive biomarker for response to programmed cell death protein-1/programmed death-ligand 1 (PD-1/PD-L1) inhibition.[5 6] However, most *POLE* mutations are either passengers with no pathogenic function or inactivate polymerase function,[7] and not all patients with *POLE* mutant tumors respond to immunotherapy. Indeed, Rousseau *et al* reported an objective

response rate (ORR) of 37% and median PFS of 5.3 months in patients with *POLE* mutated pan-cancer treated with nivolumab, and a recent phase II trial reported an ORR of 21.4% with toripalimab.[5 8] In order to generate high somatic mutation rates Pol ε must maintain its polymerase function but lose exonuclease (aka proofreading) function, resulting in error-prone synthesis. Recently, a comprehensive list of such loss-of-proofreading (LOP) *POLE* variants has been identified using the unique somatic mutation signature that results from this error-prone DNA synthesis.[1] Here we explore the implications of LOP *POLE* mutation in a large pan-cancer dataset and a cohort of patients with colorectal cancer (CRC) treated with immune checkpoint inhibitors.

METHODS

cBioPortal cohort: A pan-cancer search of 69,223 tumors on cBioPortal identified 1,965 tumors with *POLE* mutations; the search was conducted on July 6, 2023. Of these *POLE* mutant tumors, total mutation count and exome coverage were available to allow for TMB calculation of 407 tumors. A previously identified comprehensive list of LOP *POLE* variants[9] (online supplemental table 1) was used to define LOP *POLE* and non-LOP tumors.

Clinical cohort: The University of Texas MD Anderson Cancer Center Foundry database[10] was used to identify all adult patients diagnosed with metastatic CRC with *POLE* mutation. Patients were treated by CRC specialist oncologists within the Gastrointestinal Medical Oncology Department at MD Anderson, and follow-up visits, imaging, and laboratory assessment were conducted per institutional guidelines for the treatment of CRC. It is our practice to perform next-generation sequencing (NGS) molecular profiling on all patients with metastatic CRC who are candidates for systemic therapy. Targeting clinical genetic testing was performed at MD Anderson's molecular diagnostic laboratory using a clinically validated institutional multigene NGS panel, accredited by the College of American Pathologists (CAP) and certified by Clinical Laboratory Improvement Amendments. Over the course of the study, the panel was expanded from 141 to 610 genes; however, all panels included the full length (2,287 codons) of POLE.

The University of Texas MD Anderson Cancer Center internal database was used to identify all adult patients diagnosed with metastatic CRC with POLE mutation. Querying a pan-cancer molecular database identified 1,496 patients with any POLE mutation; of these, 132 had a diagnosis of CRC. Of these POLE mutant patients with CRC, 49 were treated with immune therapy between January 2017 and May 2023. 2 patients were excluded because of stage III disease and not stage IV, resulting in a cohort of 41 patients with confirmed metastatic carcinoma of colon or rectum, POLE mutation, and treated with immunotherapy (online supplemental figure 1).

Mutational landscape prevalence and enrichment analysis: Mutation profiles and associated clinical information from eight different cohorts of patients with CRC CPTAC-2 Prospective[11], CaseCCC[12], DFCI[13], Genentech[14], MSKCC (Colorectal Adenocarcinoma Triplets)[15], MSKCC (metastatic CRC)[16], MSK-IMPACT (rectal cancer)[17], and TCGA-COREAD (Firehose Legacy and Pan-Cancer Atlas)[18] were downloaded from cBioPortal (https://www.cbioportal.org/) on March 27, 2021. Mutation profiles of 1941 patients with microsatellite stable (MSS) CRC were further divided into *POLE* LOP mutants and *POLE* wild-type patients. MutSigCV (V.1.3.01) was used to identify the genes mutated significantly more frequently than random[13]; multiple hypotheses corrected threshold (q value) value of 0.1 was considered statistically significant. g:Profiler (http://biit.cs.ut.ee/gprofiler) webserver was used to perform Reactome pathway enrichment analysis on the identified list of driver genes with false discovery rate (FDR) threshold of 0.01.[19]

Statistical analysis: The Kaplan-Meier method was used for generation of survival curves with log-rank test used to test the difference in survival between groups. Overall survival (OS) was defined as the time of diagnosis until death in the cBioPortal cohort. For MD Anderson patients, progression-free survival (PFS) (including both non-immunotherapy (IO) and IO) was defined from the start of a particular therapy until its discontinuation due to radiological evidence of tumor progression or death. OS was defined as time from treatment start (either IO or first cytotoxic line analysis) to death or last contact. Median follow-up was evaluated with the reverse Kaplan-Meier method.[20] Tumor response was determined considering the most informative data available, either radiological or pathological.[21]

RESULTS

Prevalence of LOP POLE mutations

We evaluated the prevalence of LOP *POLE* mutations in a pan-cancer cohort from cBioPortal (n=69,223 samples) containing a diverse mix of 34 different tumor types. *POLE* mutation occurred in 1,965 cases (2.8%); of these, only 148 (7.5% of *POLE* mutations, 0.002% of total cohort) matched our comprehensive list of LOP *POLE* mutations. Endometrial and CRCs had the highest prevalence of any *POLE* mutation, 22.9% and 7.3%, respectively (figure 1A), consistent with prior report.[6] Although endometrial and CRCs were also the top two tumor types in terms of fraction of *POLE* mutations that cause LOP (28.7% for endometrial cancer and 18.1% for CRC), LOP mutations were still a minority (figure 1B). In terms of overall prevalence of LOP *POLE* mutations, endometrial (6.6%) and CRC (1.3%) were the only two cancer types with prevalence greater than 1% (figure 1C). LOP *POLE* mutations were concentrated in the exonuclease domain or immediately adjacent (online supplemental figure 2 A,B). In contrast, non-LOP mutations were spread throughout the coding region of *POLE* (online supplemental figure 2C).

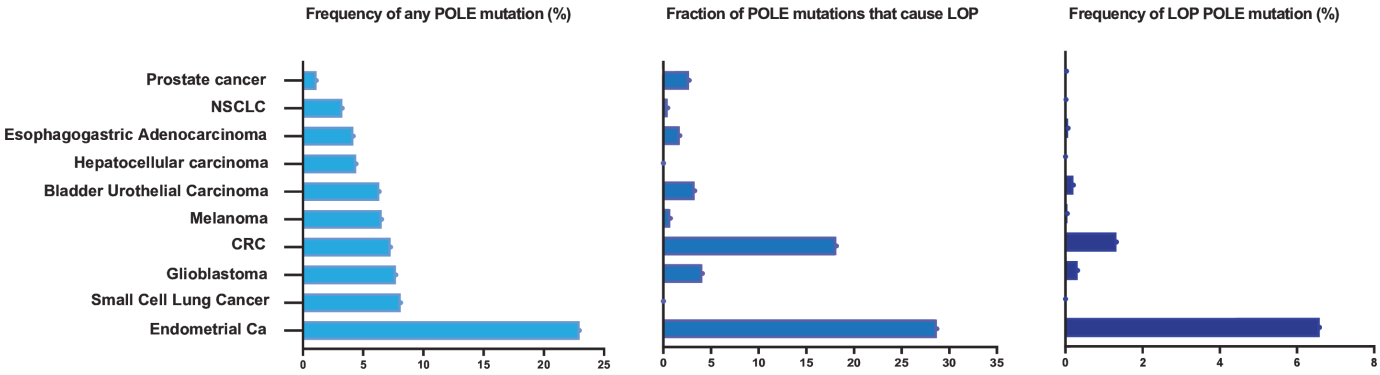

Figure 1 Epidemiology. (A) Prevalence of *POLE* mutations in different cancer types. (B) Fraction of *POLE* mutations that cause LOP by cancer type. (C) Frequency of LOP variant *POLE* mutation by different cancer type, not CRC and endometrial cancers are only tumor types with >1% incidence. CRC, colorectal cancer; LOP, loss-of-proofreading; POLE, polymerase epsilon.

LOP pole mutations have remarkably high tumor mutational burden

We found very high TMB for LOP POLE tumors in the cBioPortal cohort, over 10-fold higher compared with non-LOP *POLE* mutant tumors when filtering out tumors with other known causes of elevated TMB (microsatellite instability, smoking, and UVA exposure), with an average of 200.9 Mt/Mb (N=76) versus 18.2 Mt/Mb (N=229, median 6.2 Mt/Mb), respectively, p<0.0001; figure 2A. Clinically, TMB of 10 mut/Mb or greater is currently a US Food and Drug Administration approved indication for use of IO[22 23]; using this as a surrogate cut-off of high TMB, 74 of 76 tumors with an LOP *POLE* mutation had high TMB for a positive predictive value (PPV) of 97.4%. In contrast, if all *POLE* mutations are considered, only 81 of 229 tumors have high TMB for a PPV of 35.4%. Moreover, we also evaluated the machine learning-based algorithm PolyPhen-2, used by some molecular tumor boards to assign variant functionality (segregating them into "damaging" and "benign" categories)[24–26] to classify POLE mutations. The PPV of PolyPhen-2 to identify high TMB tumors was only 64.0% (online supplemental figure 3A). These data suggest that LOP *POLE* mutation alone is an excellent predictor of high TMB and potentially could be used by itself as a marker of immunotherapy eligibility in clinical situations when TMB data is not available.

Patients with LOP *POLE* mutations had remarkably better OS relative to wildtype (median not-yet-reached vs 63.4 months, HR=0.25, 95% CI 0.18 to 0.34, p<0.0001) as well as patients with non-LOP *POLE* mutations (not-yet-reached vs 50.7 months, HR=0.23, 95% CI 0.16 to 0.32, p<0.0001, figure 2B). In contrast, the OS of patients with non-LOP *POLE* mutations was no different than wildtype (50.7 vs 63.4 months, HR=0.94, 95% CI 0.82 to 1.07, p=0.34). Similarly, there was no survival difference between patients with *POLE* damaging mutations as defined by PolyPhen-2 compared with those defined as benign (HR=1.08, 95% CI 0.82 to 1.44, p=0.57, (online supplemental figure 3B). Endometrial adenocarcinoma was the tumor type with the greatest number of LOP *POLE* mutant tumors allowing for evaluation of survival association in a single tumor type. Patients with LOP *POLE* mutant endometrial cancer had universally favorable outcomes with 54 of 56 patients still alive with median

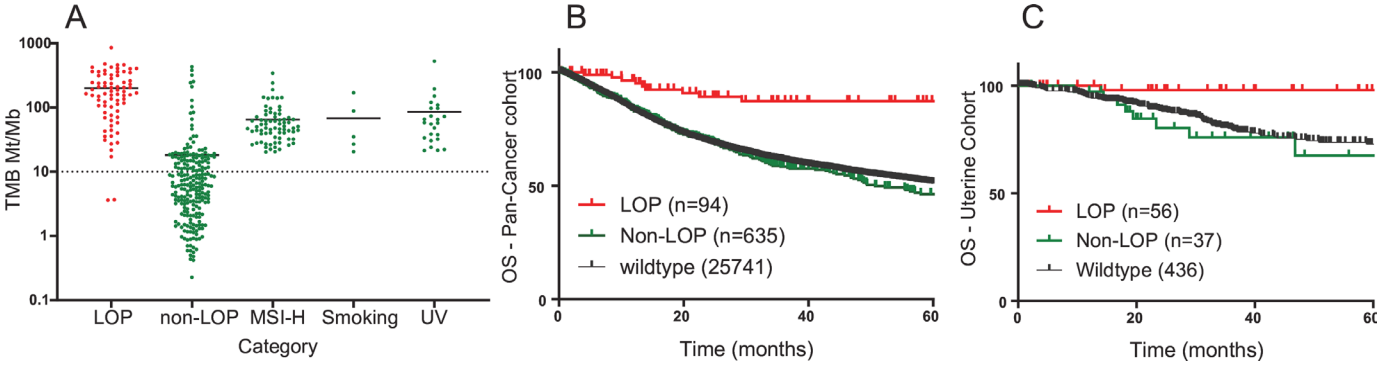

Figure 2 Identification of LOP and non-LOP mutations in the validation cohort. (A) TMB value in damaging and benign *POLE* mutations according to PolyPhen-2 dataset. (B) TMB of tumors with LOP *POLE* mutation versus non-LOP, MSI-H (microsatellite instability high), smoking and UV. (C) Kaplan-Meier investigating the survival according to LOP and non-LOP *POLE* mutation in the pan-cancer population, showing a different prognostic value according to the functionality of the alteration (p<0.0001). (D) Kaplan-Meier investigating the survival according to PolyPhen-2 definition of damaging and benign POLE mutations, showing no difference. (E) Kaplan-Meier investigating the prognostic value of LOP versus non-LOP *POLE* mutation in the uterine carcinoma subgroup showing a better outcome for LOP *POLE* mutations (p=0.0015). LOP, loss-of-proofreading; OS, overall survival; POLE, polymerase epsilon; TMB, tumor mutation burden.

Figure 3 Rate of mutations (%) of the driver genes in different groups of colorectal patients. (A) Prevalence of top 29 mutated genes in MSS LOP *POLE* tumors. (B) Prevalence of top 29 mutated genes in MSS POLE wild type tumors. (C, D) Pathway analysis performed in Reactome, showing different pathway involvement in MSS LOP *POLE* and POLE wt CRC tumors. CRC, colorectal cancer; LOP, loss-of-proofreading; MSS, microsatellite stable; POLE, polymerase epsilon; WT, wildtype.

follow-up of 37.1 months; this survival was superior relative to patients with non-LOP *POLE* mutation (HR=0.13, 95% CI 0.35 to 0.48, p=0.0015, figure 2C). Survival for patients with non-LOP *POLE* mutant endometrial cancer was not different relative to wildtype (HR=1.19, 95% CI 0.55 to 2.70, p=0.63) similar to a recent report.[27] Although a prior pan-cancer analysis of cBioPortal data suggested that any *POLE* mutation was associated with favorable OS[6], our results strongly suggested that favorable prognosis is only seen for tumors with LOP *POLE* mutations.

Mutational landscape of LOP POLE mutant CRC

In order to understand the biology that may contribute to oncogenesis in *POLE* LOP tumors, we evaluated the prevalence of co-mutated genes in *POLE* LOP CRC tumors and compared these to MSS, *POLE* wild-type CRC. Traditional co-mutation analysis cannot be performed with *POLE* LOP tumors due to *POLE* LOP causing an approximate 100-fold increase in overall mutation rate.[12 18] Apart from *APC* being the most commonly mutated gene in both cohorts, overall, the mutational landscape of LOP *POLE* mutant CRC was found to be quite distinct from MSS *POLE* wild-type CRC.[18] Although *TP53* and *KRAS* were significantly mutated in LOP *POLE* CRC, mutations in these genes were less frequent than genes such as *PDGFRA*, *TET1*, *EPHA7*, and PTEN, which were not significantly mutated in *POLE* wild-type CRC (figure 3A–B). To understand these differences at a pathway level, Reactome enrichment analysis was performed, which identified that *PI3K/AKT* signaling, CD28 co-stimulation, ubiquitin-specific protease, and defective DNA mismatch repair by *MSH2* and *MSH2* pathways were significantly enriched (FDR<0.01) in the *POLE* LOP mutant group (figure 3C). The percentage of overlap of statistically enriched pathways between LOP *POLE* and *POLE* wild-type CRC was less than random expectation (figure 3D). These differences in somatic mutation patterns suggest that the pathogenesis of LOP *POLE* CRC differs from that of wild-type *POLE* CRC following initial *APC* mutation and suggests that these tumors will have different biological properties.

Patients with LOP POLE mutant CRC have excellent response to immune therapy

The MD Anderson institutional database was queried identifying 41 patients with confirmed *POLE* mutant metastatic CRC who were treated with immune checkpoint inhibitors (online supplemental table S2). Using a comprehensive list of LOP mutations,[9] we divided the population into three cohorts: LOP *POLE* mutant, non-LOP *POLE* mutant and microsatellite instability high (MSI-H), and non-LOP *POLE* mutant and MSS. Of the nine LOP *POLE* mutation patients, only one was also MSI-H. Eight out of nine patients with LOP *POLE* mutation (88.9%) achieved an objective response including three with complete response and five with partial response; one patient had stable disease for a disease control rate of 100% (figure 4A). One patient experienced progression after 27 months while on treatment with ipilimumab and nivolumab but later achieved a pathologic complete response on an investigational BTN1A1 checkpoint inhibitor. With a median follow-up time of 32 months none of the other eight patients have progressed, and all nine patients are still alive and still being followed (figure 4B). In contrast, none of the nine patients with non-LOP *POLE* mutation and MSS tumors achieved an objective response to IO therapy with

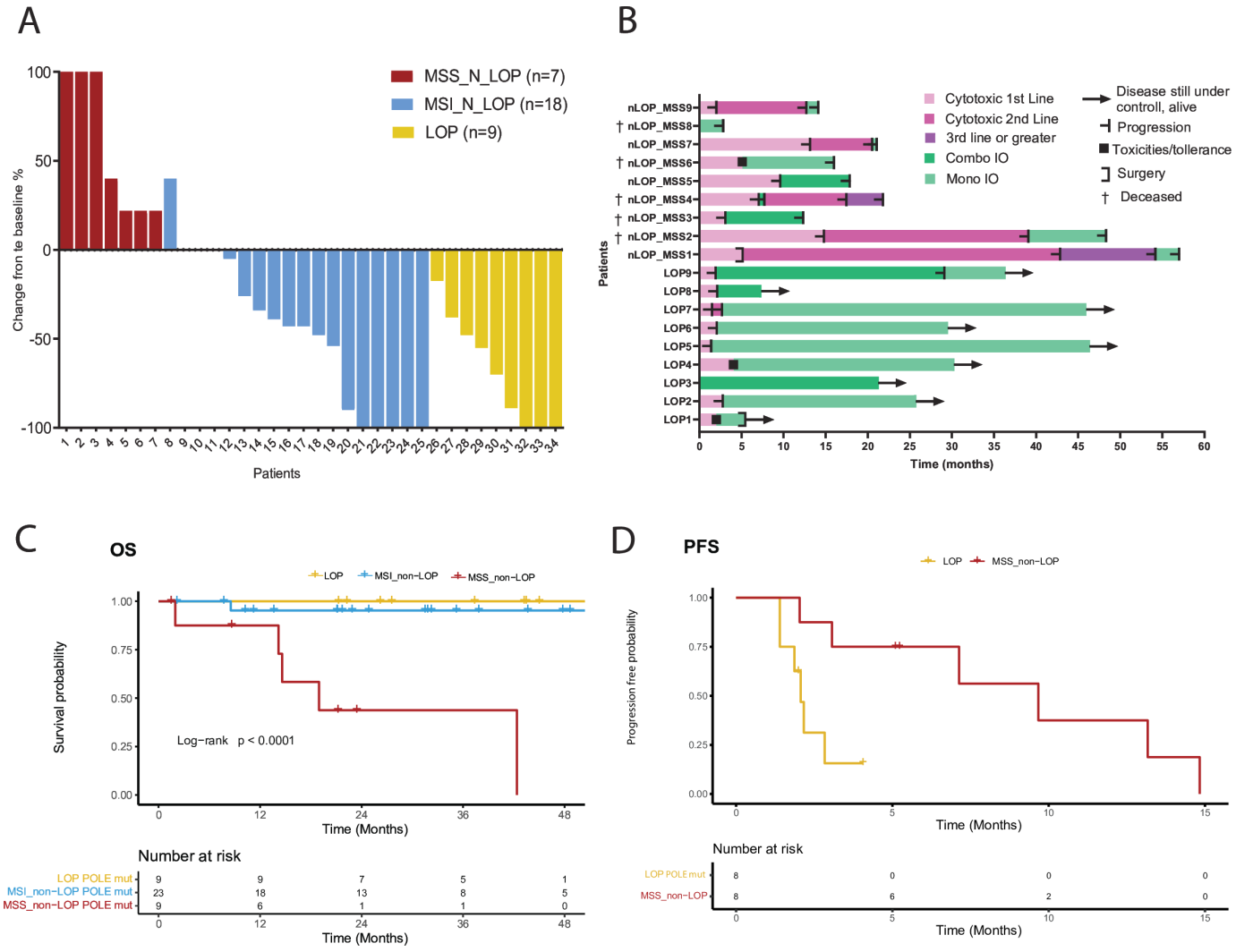

Figure 4 MDA clinical cohort. (A) Waterfall plot showing the significant response differences between different cohorts of patients. (B) Swimmer plot of LOP and non-LOP *POLE* mutated MSS patients showing the treatment duration for different class of drugs (cytotoxic vs IO), which differs between non-LOP and LOP *POLE* mutated patients. (C) Kaplan-Meier evaluation of OS in LOP-*POLE*, non-LOP-*POLE* MSS and MSI-H immunotherapy treated mCRC (metastatic colorectal cancer) subgroups. (D) Kaplan-Meier evaluation of PFS in LOP-*POLE*, non-LOP-*POLE* MSS mCRC subgroups treated with cytotoxic agents in the first-line setting. IO, immunotherapy; LOP, loss-of-proofreading; MSS, microsatellite stable; OS, overall survival; PFS, progression-free survival; POLE, polymerase epsilon.

progression at first evaluation in six cases (66.7%) and a median PFS of 3.7 months (HR 0.05, 95% CI 0.01 to 0.19, relative to LOP *POLE*, log-rank p<0.0001, online supplemental figure 4). Similarly, there was a dramatic difference in OS with median OS not yet reached for the LOP *POLE* mutant group compared with 19.0 months for MSS non-LOP patients (HR=0.05 p=0.006, figure 4C). The response to immunotherapy seen in patients with LOP *POLE* mutant tumors was superior to patients with MSI-H, non-LOP *POLE* mutant tumors, where objective response was observed in six patients (66.7%) with a median PFS not reached for both subgroups. Eight of nine patients with LOP *POLE* mutations received standard cytotoxic chemotherapy as first-line therapy (either oxaliplatin or irinotecan-based). Interestingly, median PFS for first-line chemotherapy was only 2.1 months for these patients, significantly shorter compared with the non-LOP *POLE* and MSS mutant subgroup (2.1 vs 9.7 months, HR 3.33, 95% CI 0.87 to 12.74, p=0.021, figure 4D).

DISCUSSION

POLE mutation status has been evaluated as a biomarker for patient outcome and immunotherapy response. However, there has been active debate regarding outcome stratification within *POLE*-mutated patients,[6 28] with some reports advocating that all *POLE* mutations are associated with favorable survival,[6 8] others restricting to a small number of hotspot mutations,[29 30] and others indicating all *POLE* mutations in the exonuclease domain.[31 32] Poor data are available for prognosis and treatment response in MSS non-LOP *POLE* mutant tumors and multiple active clinical trials using immune agents enroll patients with any *POLE* mutation.[33] Similarly, the recently updated

International Federation of Gynecology and Obstetrics (FIGO) staging for endometrial cancer uses a molecular-based classification which downstages early stage tumors with "pathogenic" *POLE* mutation, but does not define which mutant alleles are pathogenic.[34] These guidelines reference the *POLE*/ultra-mutated subtype of endometrial cancer identified by The Cancer Genome Atlas (TCGA);[1] however, many NGS panels, particularly those outside academic institutions, do not calculate TMB; thus, it is not possible to know if a specific *POLE* allele is associated with ultra mutation without prior knowledge. Additionally, confusion may come because mutations that disrupt the function of the entire *POLE* gene (such as frameshifts) or disrupt the polymerase function would be correctly identified as pathogenic by automated mutation assessing algorithms, as was seen with PolyPhen-2[26]; however, these mutations do not cause hypermutation. Here we highlight the difference in prognosis and treatment response between different mutant *POLE* alleles, and we demonstrate that the outcome of non-LOP *POLE* mutant is similar to *POLE* wild-type patients in both pan-cancer and endometrial cohorts, underlying the importance of correctly categorizing the proofreading functionality to address outcome prediction.[9] Given that it is not always possible to assess TMB on clinical samples, given these data, we suggest that national guidelines such as FIGO or NCCN (National Comprehensive Cancer Network) categorize mutant *POLE* alleles as either LOP or non-LOP to more precisely define patients with ultra-mutant tumors. Moreover, although a more precise definition of pathogenicity could help in alleles identification and patients' selection, incorporating into guidelines a list of variants could improve the treatment choice algorithm in clinical care, particularly outside academic institutions.

To spotlight the very relevant clinical significance of distinguishing LOP from non-LOP *POLE* mutations, we identified a cohort of patients with *POLE* mutant CRC treated with immunotherapy. To our knowledge, these 41 patients represent the largest cohort of *POLE* mutant patients treated with immunotherapy, and the only cohort in a single tumor type. We observed a deep, sustained response to immunotherapy in nine patients with LOP *POLE* in this CRC cohort, with progression only seen in one patient. This result is superior to the result of a recent pan-cancer study showing activity of immune therapy in LOP *POLE* patients but with median PFS of only 9.6 months.[5] Similar to prior reports, we found that patients with MSS CRC with non-LOP *POLE* mutations did not benefit from immunotherapy. Despite the intrinsic limitations of a retrospective and size-limited cohort, both related to the very rarity of the condition, we believe our study is presenting strong and crucial results, which should be further confirmed in prospective cohorts. Interestingly, we found that patients with CRC with LOP *POLE* mutations had particularly poor response to first-line cytotoxic chemotherapy. Although this finding should be investigated in a larger number of patients and ideally in a prospective setting, these retrospective data strongly suggest that patients with CRC with LOP *POLE* mutation should be treated with frontline immunotherapy, similar to MSI-H CRC.[35 36]

CONCLUSION

Non-LOP *POLE* mutant tumors have similar outcomes compared with *POLE* wildtype, with favorable prognosis limited to LOP *POLE* patients. Retrospective analysis of a cohort of *POLE* mutant CRC identified that patients with LOP POLE mutation had deep, sustained response to immunotherapy, and that cytotoxic agents have poor efficacy as first-line treatment for this subgroup of patients. Further investigations in bigger and prospective cohorts are strongly encouraged given our results.

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Contributors JPS, GM and FAZ conceptualized and designed the study. GM, FAZ, MAZ and AMY acquired the data. JPS, GM, SC and FAZ interpreted and analyzed the data. JPS, TAY, MJO, SK and MGW provided resources and methodology. GM, JPS, FAZ, FB, SL and SK drafted the manuscript and figures. All authors revised, edited, and approved the final version of the manuscript. JPS as guarantor accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Patient consent for publication Not applicable.

Ethics approval This retrospective study was conducted as part of the LAB09-0373 protocol (PI Kopetz), approved by the MD Anderson Institutional Review Board with a waiver of informed consent.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. Retrospective data of patients were retrieved from publicly available datasets found in cBioPortal and TCGA. Additional details of the CRC cohort can be obtained from corresponding author pending appropriate IRB approval.

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