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Basic Study

DNA polymerase epsilon-mutant colorectal cancers: Insights into non-exonuclease domain mutation variants, microsatellite instability status, and co-mutation profiles

POLE mutations in colorectal cancer

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Abstract

BACKGROUND

Although the relationship between somatic DNA polymerase epsilon (*POLE*) exonuclease domain mutations (EDMs) and colorectal cancer (CRC) is well established, the role of *POLE* non-EDMs in CRC remains unclear.

AIM

To identify *POLE* non-EDMs and EDMs in CRC, and to determine their associations with accompanying mutations and microsatellite instability (MSI).

METHODS

In this retrospective study, next-generation sequencing was performed using a targeted colon cancer panel (Qiagen, DHS-003Z) on 356 CRC patients. Of these, 191 patients were found to carry *POLE* mutations. For these patients, MSI status was assessed using both real-time PCR (EasyPGX® Ready MSI kit) and immunohistochemistry, and accompanying somatic mutations were investigated.

RESULTS

POLE mutations were identified in 53.65% of the CRC patients. Among the *POLE*-mutant patients, 87.96% were classified as pMMR (MSI-L), and 12.04% as dMMR (MSI-H). The most frequently observed *POLE* non-EDM variant was exon 34 c.4337_4338delTG p.V1446fs*3. The *POLE* EDMs were present in exon 14, with two specific variants p.Y458F (0.52%) and p.Y468N (0.52%). The most common pathogenic variants accompanying the *POLE* mutations were in *MLH3*, *MSH3*, *KRAS*, *PIK3CA*, and *BRAF* genes. *POLE* mutations were associated with a high mutational burden and MSI in CRC, particularly in the dMMR phenotype. This association suggests that *POLE* mutations may serve as important biomarkers for understanding the genetic profile of the disease and may be used in the clinical management of CRC.

CONCLUSION

This study shows a higher-than-reported prevalence of *POLE* mutations, especially non-exonuclease variants, in MSI-L CRC, highlighting their key role in tumor genetics and potential utility as biomarkers for prognosis and personalized treatment strategies.

Key Words: DNA polymerase epsilon mutation; Non-exonuclease domain variants; Microsatellite instability; Colorectal cancer; Next-generation sequencing; Somatic co-mutations

Core Tip: This study highlights the overlooked role of DNA polymerase epsilon (*POLE*) non-exonuclease domain mutations in colorectal cancer. By integrating next-generation sequencing with microsatellite instability testing, we show that *POLE* mutations are frequent, particularly in microsatellite-low tumors, and are often accompanied by co-mutations in *MLH3*, *MSH3*, *KRAS*, *PIK3CA*, and *BRAF*. These findings extend beyond the classical exonuclease domain hotspots, suggesting that both exonuclease and non-exonuclease *POLE* variants may serve as valuable biomarkers for prognosis and

support the development of personalized treatment strategies in colorectal cancer management.

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide. The genetic structure of colorectal cancers is quite heterogeneous and exhibits different mutation profiles[1]. Within this diversity, mutations occurring in the *DNA polymerase epsilon* (*POLE*) gene constitute an important subgroup[2]. The *POLE* gene is located on the long arm of chromosome 12 (12q24.33) in the human genome and spans approximately 100 kb. It comprises 49 exons and encodes the catalytic subunit of the DNA polymerase epsilon (Pol ϵ) complex, which is involved in replicative DNA synthesis[1]. *POLE* is highly expressed in tissues with elevated cell proliferation, particularly during the S phase of the cell cycle. This expression pattern underscores its critical role in DNA replication and repair processes[3]. The protein encoded by this gene consists of two major functional domains: A C-terminal 3'→5' exonuclease domain (EDM) and an N-terminal DNA polymerase domain (non-EDM)[4]. Mutations in the *POLE* gene are predominantly clustered within the EDM, spanning exons 9 to 14. These mutations are associated with a hypermutated phenotype and enhanced responsiveness to immune checkpoint inhibitors[4,5]. Notably, hotspot variants such as P286R, V411 L, and S459F impair the proofreading function of the polymerase, leading to an increased mutational burden, and may contribute to the development of microsatellite-stable (MSS) tumors that are classified as microsatellite instability (MSI) negative subtypes. This conditions may affect the immunotherapy response of *POLE*-mutant CRC patients[5-7]. Non-EDM variants may also contribute to tumor development by disrupting the structural integrity and replication fidelity of DNA polymerase ϵ . Moreover, previous studies have reported non-EDMs in different tumor types (*e.g.*, endometrial and colorectal cancer)[8,9] but their biological and clinical significance remains less well elucidated compared to EDMs, indicating the need for further investigations.

In *POLE*-mutant CRC, various co-mutations are also frequently observed in other genes. These co-mutations may affect the behavior of tumors and their responses to treatment[10]. For example, mutations are frequently observed in oncogenes and tumor suppressor genes such as *TP53*, *KRAS*, and *PIK3CA*. *TP53* mutations may accelerate tumor development by disrupting cell cycle control. *KRAS* mutations may affect the signaling pathways involved in cellular proliferation and differentiation[11-13].

In summary, while *POLE* EDM mutations are well characterized and associated with a high mutational burden and response to immune checkpoint inhibitors, the biological and clinical significance of non-EDM variants has been studied more limitedly. These variants, however, may influence tumor biology through potential effects on DNA replication and interactions with other co-mutations. Therefore, investigating non-EDMs in the Turkish CRC cohort provides important and pioneering insights by revealing population-specific mutational patterns that could contribute to personalized treatment strategies. Accordingly, this study aims to comprehensively evaluate both EDM and non-EDM *POLE* mutations in the Turkish CRC cohort, along with their MSI status and associated mutational patterns. ⁹ To the best of our knowledge, this is the first study to examine these relationships in this population, highlighting the potential clinical significance of *POLE* non-EDMs, a relatively underexplored mutation group in CRC.

MATERIALS AND METHODS

Cases

Between January 2019 and June 2024, the records of all adult patients (aged 18 and above) referred to our institutional Molecular Pathology Laboratory were retrospectively reviewed. Of the 356 consecutive patients who were diagnosed with colorectal cancer (CRC) during colonoscopies performed in the gastroenterology clinic, confirmed by histopathological evaluation, underwent next-generation sequencing (NGS) gene mutation panel analysis, and had their MSI status determined, a total of 191 *POLE*-mutant CRC patients were included in the study. Although a documented family

history of CRC was not available for all patients, none of the selected 191 *POLE*-mutant CRC cases exhibited clinical or genetic criteria indicative of hereditary CRC syndromes (e.g., Lynch syndrome or familial adenomatous polyposis). This indicates that the selected patient cohort in our study predominantly consisted of sporadic CRC cases. Patients with missing clinical data, those for whom NGS and MSI analyses could not be performed due to technical reasons, those without *POLE* mutations, and those diagnosed with hereditary CRC syndromes were excluded from the study. The inclusion and exclusion criteria for the patients enrolled in the study are summarized in Figure 1.

This study was approved by the Institutional Non-Interventional Clinical Research Ethics Committee (2024/#138), and the criteria of the Declaration of Helsinki were observed.

DNA isolation

DNA was isolated from paraffin-embedded tissue sections (10 µm in thickness) of the patients using the Qiagen GeneReader FFPE DNA isolation kit, following the kit's protocol, and conducted on the QIAcube (Qiagen Hilden, Germany) automated isolation device. Quantification and purity of the isolated DNA samples were conducted on a Qubit 3.0 fluorometer (Life Technologies, California, USA). The study proceeded with DNA samples yielding 100-150 ng of DNA.

MSI analysis

In this study, the MSI status of the patients was evaluated using both real-time PCR and immunohistochemistry (IHC), as previously described[14].

Real-Time PCR was employed to detect MSI status using the EasyPGX® Ready MSI kit, which compares the microsatellite regions in tumor tissues with those in normal tissues. This kit was used to evaluate five microsatellite loci: BAT-25, BAT-26, NR-21, NR-24, and MONO-27. Primer sequences are proprietary per the manufacturer's protocol and were not disclosed. Instability at one microsatellite locus was classified as

low MSI (MSI-L), instability at two or more loci as high MSI (MSI-H), and stability at all five loci as stable MS (MSS).

The expression of MLH1, MSH2, MSH6, and PMS2 proteins was analyzed by IHC. FFPE tissue sections were cleared with xylene, dehydrated using an alcohol series, and processed with DAKO solution at 97°C. The sections were then incubated with the appropriate antibodies for MLH1, PMS2, MSH2, and MSH6. Analyses were performed on an Autostainer Link 48 device and antigen-antibody interactions were evaluated using diaminobenzidine. Loss of nuclear staining was checked in both internal control and tumor tissues; deficient MMR proteins (dMMR) (MLH1, MSH2, MSH6, PMS2) were accepted as MSI-H, and the presence of all proteins was accepted as a proficient MMR (pMMR).

This dual approach aligns with established guidelines and ensures accurate MSI classification.

NGS analysis

Sequencing was performed using an NGS colon cancer panel (DHS-003Z, Qiagen) with the MiniSEQ NGS platform (MiniSEQ, MN00676), as described previously[14]. Sequences were aligned to the human reference genome GRCh37 (hg19). The variant analyses with NGS were conducted considering the clinical and pathological data together with the bioinformatics support from the Clinical Insight Interpret software (8.1.202021, Qiagen). Variants were filtered based on read depth $\geq 250x$, variant allele frequency $\geq 5\%$, and annotation confidence. Variants were divided into four categories according to cancer diagnosis, prognosis, and treatment effects.

Data analysis

4 Analysis of the data was performed using SPSS 22.0 (IBM, Armonk, NY, USA). χ^2 test was utilized for comparing the 10 categorical data. The significance level was set at $P < 0.05$. The results are presented as numbers and percentages.

RESULTS

A *POLE* mutation was observed in 191 (53.65%) of 356 CRC patients. All clinical and pathological features of *POLE*-mutant CRC patients are presented in Table 1.

The most frequently observed variation in the *POLE* gene in patients was the non-EDM variant exon 34 c.4337_4338delTG p.V1446fs*3 (conflicting classifications of pathogenicity), identified in 182 patients (95.29%) (Table 2). Other observed *POLE* non-EDM variants in the patients included p.D612N (0.52%), p.R1909 (0.52%), p.N518fs*10 (0.52%), p.Q1774* (0.52%), p.Q911* (0.52%), p.A1885T (0.52%), and p.L1171fs*6 (0.52%). *POLE* EDM (exons 9-14) mutations were rare, and only p.Y458F (0.52%) and p.Y468N (0.52%) variants located in exon 14 were detected in patients (Table 2).

The 10 most frequently accompanying mutations to *POLE* mutations were pathogenic mutations in the *MLH3* (72.25%), *MSH3* (72.25%), *KRAS* (41.36%), *PIK3CA* (14.66%), *BRAF* (6.81%), *PMS2* (5.76%), *TP53* (5.76%), *MSH6* (5.24%), *ERCC5* (5.24%), and *TCF7 L2* (5.24%) genes (Figure 1). All gene variations accompanying *POLE* mutations in the subjects are listed in Table 3.

In the MSI IHC evaluation of *POLE*-mutant patients, 168 (87.96%) cases were classified as pMMR (MSI-L), whereas 23 (12.04%) cases were classified as dMMR (MSI-H). In the MSI evaluation using real-time PCR, 165 cases were identified as MSI-L, 8 cases as MSS, and 18 cases as MSI-H. The real-time PCR and IHC results showed approximate concordance.

Table 4 provides stratified descriptive statistics of *POLE*-mutant CRC patients by age, tumor location, and MSI status, including the most frequent co-mutations. EDM mutations were extremely rare ($n = 2$), whereas the majority of *POLE* mutations were non-EDM ($n = 189$). Among *POLE*-mutant patients, MSI-H was observed in 23 cases (12.04%), primarily among non-EDM mutations. *POLE* mutations were more common in the colon than in the rectum across all MSI categories. Age stratification showed that most patients were between 50–65 years old. The estimated odds ratio (OR) for MSI-H in EDM *vs* non-EDM cases was 0.03 (95%CI: 0.001–1.2, $P = 0.06$), highlighting the low prevalence of EDM mutations and the need for cautious interpretation. Co-mutation

patterns, most frequently *MLH3*, *MSH3*, *KRAS*, *PIK3CA*, *BRAF*, *PMS2*, *TP53*, *MSH6*, *ERCC5*, and *TCF7 L2*, were mainly observed in non-EDM cases.

DISCUSSION

This study is the first to investigate *POLE* EDMs, non-EDMs, and accompanying mutations in Turkish patients with CRC, along with the MSI status of patients.

POLE mutations are generally considered significant genetic findings in various cancer types, including CRC. Over the past decade, research has shown that somatic mutations in the *POLE* gene are present in 1-12.3% of CRCs, with *POLE* EDMs being present in only 1-2% of cases[8,10,15]. For example, in a study by Domingo *et al*[15], *POLE* mutations were reported in only 1% of CRC patients. Similarly, Guo *et al*[10] reported an incidence of 1.5% for *POLE* mutations in a Chinese cohort of CRC patients. However, both studies emphasized that these mutations contribute significantly to the genetic profile of the disease and are associated with a high mutational burden. The most striking observation in the current study was that *POLE* mutations were present in 53.65% of CRC patients in the Turkish cohort, a percentage much higher than that reported in other populations. This figure is particularly noteworthy when compared with the findings in literature. The markedly higher *POLE* mutation rate observed in our study (53.65%) compared to previous global reports is likely attributable to referral bias, since NGS was predominantly requested for consecutive CRC patients with advanced disease or suspected molecular alterations. Therefore, our findings should be interpreted with caution, as they may not represent the broader Turkish CRC population. Multicenter studies with more diverse cohorts are warranted to validate these observations.

Despite a lack of sufficient evidence to support the pathogenic role of *POLE* non-EDMs, few studies have validated their pathogenicity[16]. Stenzinger *et al*[8] identified somatic *POLE* non-EDMs in 12.3% of sporadic MSS CRC cases. Furthermore, another study found *POLE* non-EDMs in 3-4% of CRCs and endometrial cancers[9]. Additionally, consistent with the literature, our study identified a *POLE* EDM rate of

approximately 1%, while the incidence of *POLE* non-EDMs was found to be higher than that previously reported. This elevated rate may be attributed to population-specific characteristics or differences in the genetic analysis methods.

In this study, the most frequently observed variation was the exon 34 c.4337_4338delTG p.V1446fs*3 frameshift variant, classified as "Conflicting classifications of pathogenicity." This specific mutation has not been previously reported in CRC patients. Previous studies, such as Stenzinger *et al*[8] and Briggs *et al*[9], have reported different non-EDM mutations in CRC and endometrial cancer cohorts. This mutation represents the first *POLE* non-EDM variant with a high incidence in Turkish CRC patients and is generally associated with a high mutational burden. Our study also reports the frequency of other *POLE* non-EDM and EDM variants; gaining further insight into the pathogenic effects of these variants is particularly important for informing therapeutic approaches. Although our findings expand the mutational spectrum of *POLE* in CRC, larger-scale studies including different Turkish CRC subgroups, as well as functional analyses, are warranted to clarify the biological impact and generalizability of this variant.

Excluding potentially recurrent mutations in CRC, other distinct variants have also been listed in the literature for *POLE*. Most of these variants are present in the EDM and include mutations, such as p.W347C, p.N363Ks, p.D368V, p.K425R, p.P436S, and p.Y458F[17]. Among them, p.D368V and p.Y458F were functionally verified[18-22]. In the current study, *POLE* EDMs identified in patients included the p.Y458F and p.Y468N variants. The p.Y458F variant has been classified as a class 5 pathogenic variant of CRC, as reported by Rocque *et al*[23]. The p.Y468N variant (classified as "uncertain significance") identified in our patients represents the first reported *POLE* EDM in the Turkish population. However, the frequency of EDMs was lower than that of the non-EDMs. This discrepancy could be due to population differences and the limited number of studies that detected non-EDMs.

The incidence of somatic *POLE* mutations ⁷ has been reported to be higher in patients with colon cancer than in those with rectal cancer[24,25]. Similarly, in our study, the mutation incidence was higher in colon cancer, which is consistent with the literature.

The frequency of dMMR/MSI-H tumors in patients with CRC is approximately 15-20%, with stage IV dMMR/MSI-H tumors representing only 2-4% of all metastatic CRC cases[26]. In our study, a similar rate was observed, with 12.04% of CRC patients showing a dMMR/MSI-H status, which is consistent with literature.

The identification of 87.96% of *POLE*-mutant patients as having MSI-L indicates a strong association between these mutations and MSI-L. Carethers *et al*[27], *POLE* mutations are typically associated with an ultra-mutated phenotype but not necessarily with high microsatellite instability (MSI-H), which is consistent with our finding that most *POLE*-mutant tumors were MSI-L.

In a study conducted by Lin *et al*[26], MSI-H CRCs were associated with a higher incidence ¹ of mutations in *BRAF*, *PIK3CA*, and *PTEN* genes, as well as in receptor tyrosine kinase families. Conversely, ¹ MSS CRCs exhibited a higher incidence of mutations in the *APC*, *KRAS*, and *TP53* genes. In our study, despite the findings of Lin *et al*[26], both MSI-H and MSI-L patients with CRC had similar gene mutation profiles. This may be attributed to population differences.

In our cohort, the frequent co-occurrence of *POLE* mutations with variants in *MLH3*, *MSH3*, *KRAS*, *PIK3CA*, and *BRAF* is noteworthy. These co-mutation patterns point to several possible biological mechanisms. DNA repair pathways (MMR genes: *MLH3*, *MSH3*, *PMS2*, *MSH6*): Co-mutations in these genes, together with *POLE* alterations, may exacerbate DNA repair deficiencies, driving tumors toward an ultra-mutated phenotype. This may, in turn, enhance sensitivity to immune checkpoint inhibitors[2,15,16,24]. Oncogenic signaling pathways (*KRAS*, *PIK3CA*, *BRAF*): Mutations in the MAPK and PI3K/AKT pathways, when combined with the mutational burden induced by *POLE* alterations, may increase immunogenicity and tumor heterogeneity[26,27]. Tumor suppressor genes (*TP53*, *PTEN*, *SMAD4*): Additional mutations in these genes may contribute to loss of cell cycle control and the

development of a more aggressive tumor phenotype[15,27]. These patterns indicate that *POLE* mutations shape tumor biology not in isolation, but in concert with co-mutations. Clinically, such combinatorial mutation profiles may help to identify patient subgroups most likely to benefit from immunotherapy and may guide the discovery of novel therapeutic targets[16,24,26].

Our study not only highlights the high frequency of *POLE* mutations but also emphasizes the importance of their associated co-mutation profiles. Evaluating *POLE* mutations in conjunction with MSI status and co-mutations may help better identify candidates for immunotherapy[15,16]. Although the pathogenic effects of *POLE* non-EDMs have not been fully established, their high prevalence observed in our study suggests potential impacts on CRC biology. The frequent co-occurrence of these mutations with key genes such as *KRAS* and *PIK3CA* may influence tumor immunogenicity and supports the exploration of combination therapeutic approaches (e.g., immunotherapy plus targeted therapies)[26]. Even without functional validation, these findings provide a foundation for future studies aimed at assessing the prognostic and therapeutic significance of non-EDMs.

Limitations

This study has several limitations. The retrospective design introduces a potential risk of selection bias. In particular, since NGS was more frequently requested for advanced CRC cases, the frequency of *POLE* mutations may appear higher than it truly is. The cohort consisted solely of CRC patients from a single center in Türkiye, which limits the generalizability of the findings to broader populations. The functional impact of *POLE* non-EDM variants has not been experimentally validated in this study. Moreover, the *POLE*-mutant subgroup was relatively small ($n = 191$), with a limited number of patients in the MSI-H subset. Finally, the absence of long-term follow-up data precluded evaluation of the prognostic impact of these mutations or their association with treatment responses.

CONCLUSION

This study demonstrates that the overall *POLE* mutation rate and the frequency of *POLE* non-EDMs in MSI-L CRC are significantly higher than previously reported. Therefore, *POLE* mutations may be considered as important genetic biomarkers in CRC. The association of these mutations with high mutation load and MSI provides critical information for clinical evaluation and treatment strategies. In addition, the presence of *POLE* mutations plays a crucial role in understanding patients' genetic profiles and developing personalized treatment strategies. The observation that *POLE* non-EDMs may be more prevalent requires further validation. If confirmed, this finding could increase the number of patients who could benefit from immune-based therapy.

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