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Tumor Discovery

ORIGINAL RESEARCH ARTICLE

Exploring somatic mutations in *POLE* and *POLD1*: Their role in colorectal cancer pathogenesis and potential therapeutic strategies

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Abstract

Colorectal cancer (CRC) is one of the major causes of morbidity and mortality worldwide, resulting from the accumulation of genetic and epigenetic alterations in several oncogenes and tumor suppressor genes. Recent studies have identified germline and somatic mutations in the exonuclease domain regions of both epsilon polymerase (*POLE*) and delta polymerase (*POLD1*) genes in CRCs. We sought to examine the mutation of these genes in a series of sporadic CRCs. To do this, we extracted DNA from 100 primary CRC samples and 40 corresponding normal tissue samples, which had been previously characterized for clinicopathological and molecular features. We employed a combination of quick-multiplex consensus (QMC)-polymerase chain reaction (PCR) and co-amplification at lower denaturation temperature (COLD)-PCR, followed by high-resolution melting (HRM) analysis and Sanger sequencing, to investigate the exonuclease domain regions of *POLE* and *POLD1* genes for somatic mutations that may potentially alter the proofreading activities of these genes. *In silico* predictions of the functional significance of the identified genetic alterations were performed using the protein variation effect analyzer, sorting intolerant from tolerant, and PON-P2 algorithms. We identified a total of eight new and non-recurrent somatic variants in the endonuclease domains of *POLE* and two in *POLD1*. Nine out of ten variants caused amino acid substitutions, whereas one resulted in a stop codon. Although no significant associations or correlations were found between the *POLE/POLD1* mutations and the clinicopathological or molecular features of the CRC cases, most of the *POLE/POLD1*-mutated cases were microsatellite stable (90%) and aneuploid (80%). Furthermore, *in silico* analyses showed that nine of the ten variants would likely cause some adverse effect on protein function. Ten somatic variants with predicted proofreading activity-altering effects have been identified in the endonuclease domains of *POLE* and *POLD1* in CRC using a combination of QMC-PCR, COLD-PCR, HRM analyses, and Sanger sequencing.

Keywords: Colorectal cancer; Novel somatic mutations; *POLE*; *POLD1*

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1. Introduction

Colorectal cancer (CRC) is a complex disease driven by various genetic alterations that influence its initiation and progression.[1] It ranks as the fourth most common cancer in both men and women. The majority of CRC cases – approximately 70 – 80% – are sporadic. In contrast, about 20 – 30% have a hereditary component, resulting from either rare, high-risk genetic tumor syndromes such as Lynch syndrome (3 – 4%) and familial adenomatous polyposis (about 1%) or from more common but lower-risk alleles identified through genome-wide association studies. Only 1 – 2% of CRC cases are associated with inflammatory bowel diseases.[2] Among these alterations, impairments in DNA replication fidelity, particularly due to mutations in the proofreading domains of DNA polymerases ε (*POLE*) and δ (*POLD1*), have garnered significant attention.[3] Wang *et al.*[4] revealed that somatic mutations in *POLE* and *POLD1* occurred at rates of 2.79% and 1.37%, respectively.

Both the *POLE* and *POLD1* play crucial roles in ensuring accurate DNA replication and repair, making them integral components of cellular genomic integrity. Germline mutations in these genes have been associated with an increased risk of CRC development, implicating their involvement in hereditary CRC syndromes.[5,6] For example, mutations such as *POLE* P286R and *POLD1* S478N have been identified in familial CRC cases, leading to an elevated mutation burden and distinct mutational signatures.[7,8] Furthermore, somatic mutations in *POLE* and *POLD1* have been observed in sporadic CRC cases, contributing to tumor heterogeneity and progression. These somatic mutations are often associated with microsatellite stability (MSS) in CRC tumors, influencing their mutational landscape.[9-11]

The functional consequences of *POLE* and *POLD1* mutations extend beyond increased mutational burden. For instance, *POLE* V411L and *POLD1* C319Y have been linked to a hypermutator phenotype, resulting in an increased neoantigen load and potential implications for immunotherapy response.[3,12] Understanding the implications of both germline and somatic mutations in *POLE* and *POLD1* is crucial for advancing our knowledge of CRC pathogenesis and guiding personalized therapeutic approaches.[13] These mutations not only provide insights into CRC etiology but also hold promise as biomarkers for risk assessment and treatment stratification.[14]

The aim of the present study is to investigate the rate of somatic mutations in the exonuclease domains of both *POLE* and *POLD1* genes in a well-characterized cohort of CRC and to define the clinicopathological and molecular characteristics of *POLE* and *POLD1*-mutated cancers.

2. Materials and methods

2.1. Sample population

One hundred formalin-fixed and paraffin-embedded (FFPE) primary CRC samples had been previously retrieved from the archives of the pathology department of the Nottingham University Hospitals NHS Trust. The CRC tissue samples were accompanied by 40 cases of matched normal mucosa, each carefully located away from the tumor site and processed in separate blocks.

All patients had undergone surgery between 2003 and 2010 at Queen's Medical Center, Nottingham, United Kingdom (UK). The cases were selected based on the availability of clinicopathological data and the presence of at least 50% of tumor tissue in the biopsy specimens and tumor block (Table 1).

The ploidy and microsatellite status of all samples were previously determined by Fadhil *et al.*[15] In brief, ploidy status was examined using flow cytometry, whereas the microsatellite status of the cases was determined by polymerase chain reaction (PCR) followed by high-resolution melting (HRM) evaluation of instability at mononucleotide repeat sequences using a panel of 6 markers (BAT25, BAT26, NR21, NR22, NR24, and B-CAT25).

2.2. DNA extraction

Six 10 mm sections were cut from each block, and DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen, UK) following the manufacturer's protocol, as previously described.[16]

2.3. Primer design and optimization

The *POLE* and *POLD1* nested primers were designed for use in quick-multiplex consensus (QMC)-PCR assays, as previously described.[17] The genomic sequences of the exonuclease domains of *POLE* (codons 268 – 471) and *POLD1* (codons 304 – 517) were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/pubmed/). Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) was

Table 1. Comparison of the clinicopathological features of patient cohorts

Variable	Classification	Cohort, *n*=102; frequency, *n* (%)	Cohort, *n*=83; frequency, *n* (%)
Sex	Male	57 (56)	49 (59)
	Female	37 (36)	34 (41)
	Unknown	8 (8)	
Age	Median	70 (43 – 88)	71 (43 – 80)
Dukes' stage	A	6 (6)	4 (5)
	B	29 (28)	29 (35)
	C	41 (40)	39 (47)
	D	2 (2)	11 (13)
	Unknown	24 (23)	0
Vascular invasion	V0	49 (48)	42 (51)
	V1	31 (30)	29 (35)
	V2	1 (1)	1 (1)
	Unknown	21 (21)	11 (13)
Tumor stage	pT1	3 (3)	3 (4)
	pT2	6 (6)	3 (5)
	pT3	47 (46)	43 (52)
	pT4	24 (23)	21 (25)
	Unknown	22 (22)	12 (14)

used to design outer and inner primers for all the targets of interest, including exons 9 – 14 of *POLE* and exons 8 – 13 of *POLD1*. All the inner primers were designed to amplify PCR products that are <250 bp. In total, six sets of outer and eight sets of inner primer pairs were designed for *POLE* and *POLD1*, respectively. Primer specificities were assessed using UCSC *in silico* PCR (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start), as well as on MFEprimer-2.0 (http://biocompute.bmi.ac.cn/CZlab/MFEprimer-2.0/index.cgi/check_dimer), which also excluded possible primer-dimer formations. The optimal annealing temperatures for all primers were determined through gradient PCR and gel electrophoresis on a Primus PCR machine. The annealing temperature for all eight *POLD1* outer primer pairs was set at 60°C, whereas all eight *POLD1* inner primers and all the *POLE* primers (six sets of outer and inner primer pairs) had an annealing temperature of 55°C. The gradient PCR cycling conditions were as follows: one cycle at 95°C for 5 min; 40 cycles of denaturation at 95°C for 10 s, annealing at 55 ± 5°C for 30 s, and extension at 72°C for 20 s (refer Tables A1 and A2 for the outer and inner primer sequences). The specificities of the individual inner primers were further verified using standard PCR followed by gel electrophoresis (Figures A1 and A2).

2.4. Quick-multiplex consensus-PCR protocols

2.4.1. Thermal cycling conditions

The QMC-PCR was performed as previously described.[16] The pre-diagnostic multiplex (PDM) reaction was performed in a final volume of 25 µL, containing 1× HotShot master mix (Cadama Medical Ltd., UK), 250 nM of each primer, and 20 ng template DNA. The PDM reactions were carried out separately for the *POLE* and *POLD1* targets. For the *POLE* primers, PCR was performed using a two-step cycling protocol: One cycle at 95°C for 5 min, followed by 25 cycles of 95°C for 1 s and 55.5°C for 1 s; whereas for the *POLD1* targets, the PCR cycling was 3-step: 1 cycle at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 10 s. The single specific diagnostic (SSD) reaction was performed in a final volume of 10 µL, containing 1× HotShot master mix, 1 primer pair (each primer at 250 nM final concentration), 1× LC Green PLUS (Idaho Technology, United States), and water to complete the total volume. The template consisted of 1 µL of a 1:100 dilution of the PCR product from the PDM reaction. The PCR was performed using a two-step protocol: 1 cycle of denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 1 s, and annealing at 55°C for 1 s.

2.4.2. Co-amplification at lower denaturation temperature (COLD)-PCR

Full co-amplification at a lower denaturation temperature PCR followed by HRM (full COLD-PCR-HRM) analysis was used to enrich for minor alleles that were below the detection limits of Sanger sequencing.[18] First, the critical temperatures (Tc) for selected *POLE* and *POLD1* targets were determined using gradient PCR with the following parameters: 1 cycle of initial denaturation at 95°C for 5 min; 10 cycles of denaturation at 95°C for 1 s and annealing at 55.5°C for 1 s; followed by 25 cycles of denaturation at Tm ± 4°C for 10 s, annealing at 55°C for 15 s, and extension at 72°C for 10 s. Following HR-1 HRM, the lowest denaturation temperature that produced amplification was chosen as the Tc for each target. The cycling parameters for the full COLD-PCR were as follows: 1 cycle of initial denaturation at 95°C for 5 min; 45 cycles of denaturation (95°C/30 s), hybridization (70°C/30 s), denaturation (Tc/30 s), annealing (55.5°C/1 min), and extension (72°C/1 min); with 1 cycle of final extension at 72°C for 10 min.

2.5. HRM analysis

The PCR products of the SSD reactions were transferred to light cycler capillaries (20 µL) (Roche, Switzerland). The products were loaded in the HR-1 HRM instrument (Idaho Technology) and melted by raising the temperature at a

rate of 0.3°C/s, with a starting temperature of 70°C, a final temperature of 95°C, and fluorescence being measured between 80 and 95°C. The HR-1 analysis tool and custom software were used to analyze all the data, and both derivative plots and difference plots were generated after normalizing and temperature shifting. A 4% difference in fluorescence between the known wild-type control and test samples was used as the threshold for the variant call, as previously described.[2]

2.6. Sequencing

The PCR products of tumor samples with abnormal HRM patterns were subjected to direct, bidirectional Sanger sequencing. First, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Netherlands) according to the manufacturer's instructions. The purified products were then sequenced directly using the appropriate PCR primers. The resulting chromatograms were viewed and interpreted using Chromas Lite software V2.01. To analyze the sequencing data, the Basic Local Alignment Search Tool 2 sequences program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) was used to compare the sequences against the wild-type sequences for both *POLE* and *POLD1* genes.

2.7. Statistical analysis

The clinicopathological and molecular significance of the *POLE* and *POLD1* mutations were tested using SPSS version 22. Categorical data were analyzed using the Chi-squared test (or Fisher's exact test, when the expected count in each cell was <5), whereas continuous variables were analyzed using the independent *t*-test. The results were significant at $P < 0.05$.

2.8. Prediction of the functional effects of mutations

The batch protein function of the protein variation effect analyzer (PROVEAN) online software (http://provean.jcvi.org/) and the PON-P2 computational tool (http://structure.bmc.lu.se/PON-P2/) were used to predict the possible functional effects of the *POLE* and *POLD1* mutations.[19-21] The identification numbers of *POLE* and *POLD1* were retrieved from ENSEMBL (ENSP number, www.ensenbl.org), NCBI (RefSeq number, www.ncbi.nlm.nih.gov/protein), and UniProt (SwissProt protein identifier number, www.uniprot.org) and inputted together with the protein positions and amino acid substitutions into the PROVEAN and PON-P2 software.[19,20] The PROVEAN batch protein software displays results of both the PROVEAN and SIFT (Sorting Intolerant From Tolerant, another protein function prediction software, http://sift.jcvi.org) algorithms.[21] Mutations that would likely cause alterations in protein functions are called "deleterious" (PROVEAN), "damaging" (SIFT), or "pathogenic" (PON-P2), whereas those unlikely to result in functional changes are displayed as "neutral" (PROVEAN, PON-P2) or "tolerant" (SIFT). The PON-P2 computational tool also returns a prediction call of "unknown" if the effect of the amino acid substitution is indeterminate.[20]

3. Results

DNA from 102 tissue samples was tested for mutations in the endonuclease domains of *POLE* and *POLD1* with optimized QMC-PCR followed by HRM analyses. The HRM analysis results were confirmed by Sanger sequencing. When Sanger sequencing failed to confirm aberrant HRM analysis results, the samples were amplified with COLD-PCR and then re-sequenced for confirmation.

3.1. *POLE* and *POLD1* endonuclease domain mutations

A total of 10 novel somatic variants (eight in *POLE* and two in *POLD1*) were found in ten different cases, giving a variant rate of 9.6% and 2.4% in *POLE* and *POLD1*, respectively, in the entire cohort of patients. Nine of these

Table 2. Molecular characteristics of *POLE* and *POLD1* mutations

Subject id.	Gene/Exon	Nucleotide position	Codon	Functional amino acid change	Microsatellite status	Ploidy status
4	*POLE*/14	c. 1364 T>A	L455Q	Hydrophobic to polar	MSS	A
32	*POLE*/9	c. 833 C>T	T278M	Hydrophilic to hydrophobic	MSS	A
38	*POLE*/13	c. 1295 T>C	L432P	Hydrophobic to hydrophobic	MSS	A
40	*POLE*/13	c. 1240 G>A	D414N	Polar to polar	MSS	D
44	*POLE*/12	c. 1121 C>T	A374V	Hydrophobic to hydrophobic	MSS	A
63	*POLE*/13	c. 1298 G>A	G433D	Hydrophilic to polar	MSS	A
64	*POLE*/14	c. 1369 A>G	T457A	Polar to hydrophobic	MSS	A
83	*POLE*/14	c. 1382 C>A	S461*	Truncated protein	MSI	D
50	*POLD1*/10	c. 1211 C>T	P404L	Hydrophobic to hydrophobic	MSS	A
52	*POLD1*/10	c. 1229 C>T	A410V	Hydrophobic to hydrophobic	MSS	A

Abbreviations: MSI: Microsattelite instability; MSS: Microsattelite stable.

variants were non-synonymous variants, which resulted in amino acid substitutions in the endonuclease domains of *POLE* and *POLD1*. One mutation in the *POLE* resulted in a stop codon, which is predicted to result in pol ε translation termination. With the exception of the *POLE* D414N variants, all the other variants in *POLE* and *POLD1* were found in the primary tumors (Table 2). However, no variant was found in the corresponding normal tissues of these cases, thereby confirming that these novel alterations were somatic mutations rather than germline SNPs. Sanger sequencing data also validated the novel alterations as somatic and not germline (Figures 1A-F).

No recurrent mutations or double heterozygous mutations were identified among the new *POLE/POLD1* alterations. Furthermore, the previously described germline *POLE* L424D or *POLD1* S478N mutations were not detected in our cohort. In our previous study,[22] we performed NGS analysis on the same cohort of samples that was used here. Based on our findings, only four out of ten samples with mutations in the POLE and POLD1 genes exhibited a hypermutated phenotype. These samples were more likely to have a full complement of mutations in APC, KRAS, SMAD4, and TP53. In contrast, the remaining six out of ten samples were more likely to have subclonal mutations (Table 2).

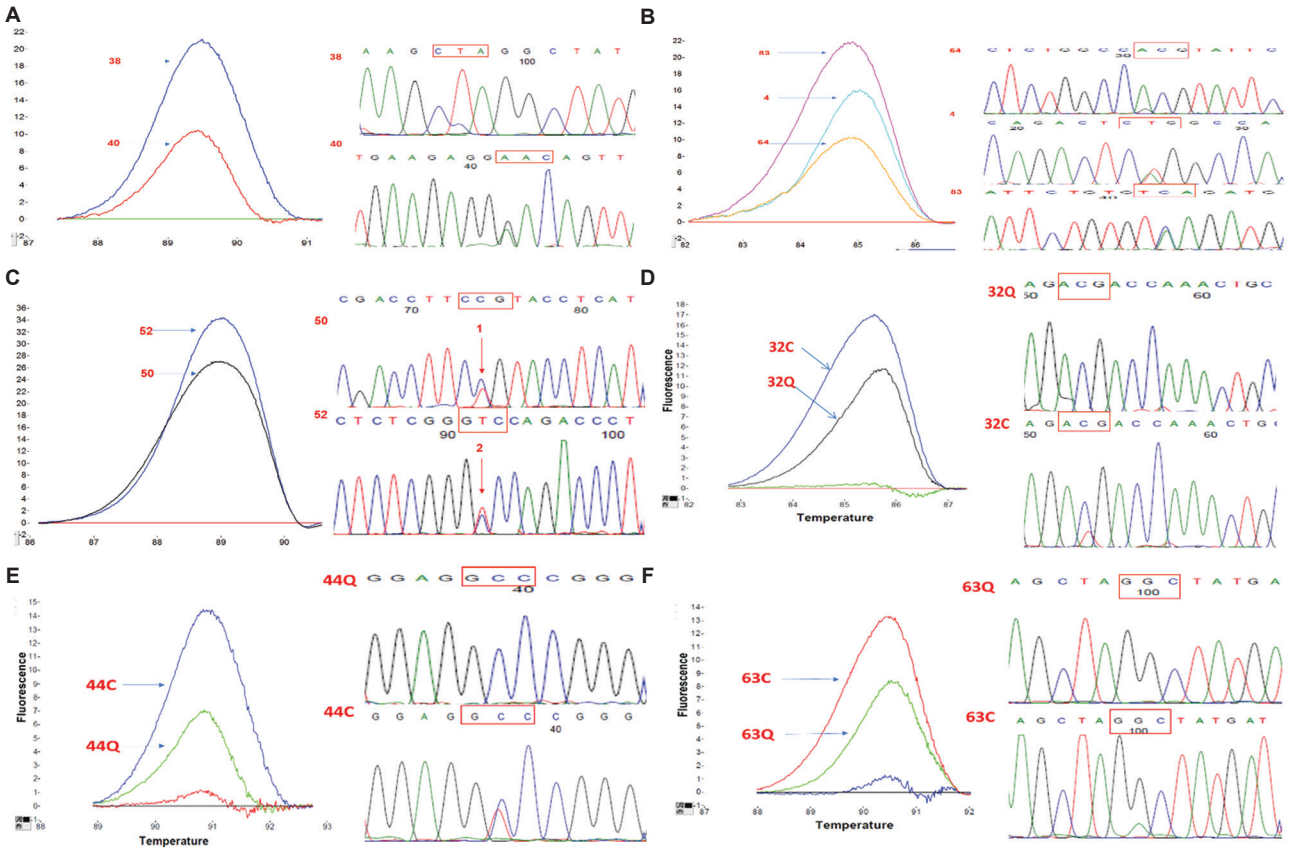


Figure 1. High-resolution melting (HRM) analysis screening and validation by Sanger sequencing of the *POLE* and *POLD1* mutations in FFPE CRC samples from the Nottingham cohorts. (A) Difference plots showing aberrant melting patterns of samples 38 and 40 at the *POLE* exon 13. Sanger sequencing confirms codon 432 CTA to CCA and codon 414 GAC to AAC *POLE* exon 13 mutations in samples 38 and 40, respectively. (B) Difference plots showing aberrant melting patterns of samples 64, 4, and 83 at the *POLE* exon 14. Sanger sequencing confirms the exons 14 codons 457 ACG to GCG, codon 455 CTG to CAG, and the codon 461 TCA to TAA mutations in samples 64, 4, and 83, respectively. (C) Difference plots of samples 38 and 40 show aberrant melting patterns at the *POLD1* exon 10. These mutations were confirmed to be codon 404 CCG to CTG and the codon 410 GCC to GTC mutations in POLD1 exon 10 in samples 50 and 52, respectively, by sequencing. (D) Difference plots showing enrichment of the codon 278 ACG to ATG mutation of *POLE* exon 9 in sample 32 by COLD-PCR and validation by Sanger sequencing. 32C = COLD-PCR-amplified sample 32; 32Q = QMC-PCR-amplified sample 32. (E) COLD-PCR enrichment of codon 374 GCC to GTC *POLE* exon 12 mutation in sample 44 (as seen in HRM Difference plots) was validated by sequencing. 44C = COLD-PCR-amplified sample 44; 44Q = QMC PCR-amplified sample 44. (F) Difference plots showing COLD-PCR enrichment of the codon 433 GGC to GAC mutation in *POLE* exon 13. The mutant allele enrichment was confirmed by sequencing by the Sanger method (63C = COLD-PCR-amplified sample 63; 63Q = QMC-PCR-amplified sample 63)

Abbreviations: COLD: Co-amplification at lower denaturation temperature; CRC: Colorectal cancer; FFPE: Formalin-fixed and paraffin-embedded; QMC: Quick-multiplex consensus; PCR: Polymerase chain reaction.

3.2. Clinicopathological and molecular significance of *POLE/POLD1*-mutations in CRC

There was no significant association or correlation between *POLE/POLD1* variants and any of the clinicopathological and molecular features examined (Table 3). However, 90% of the *POLE/POLD1*-mutated tumors were microsatellite stable, and 80% of them were also aneuploid. This lack of association was observed irrespective of whether *POLE* and *POLD1* were analyzed separately or together.

3.3. Functional effects of *POLE* and *POLD1* variants

The possible effects of the *POLE* and *POLD1* variants on the proofreading activities of the enzymes were tested *in silico* using the PROVEAN batch protein and the PON-P2 protein prediction tools. The results showed that whereas both variants identified in *POLD1* could confer possible "deleterious," "damaging," and "pathogenic" effects on the protein by all of PROVEAN, SIFT, and PON-P2 predictions, respectively, four of the *POLE* variants (L455Q, T278M, L432P, and D414N) were predicted to be deleterious/damaging/pathogenic by all three prediction tools, one (A374V) was scored neutral/tolerated by all three, whereas two (G433D and T457A) were predicted to be damaging or pathogenic by at least one prediction tool (Table 4). The *POLE* exon 14 codon 461 variant identified gives rise to a stop codon, which we considered to have a deleterious/damaging effect on protein translation. In total, nine of the 10 *POLE/POLD1* variants identified were predicted to alter the proofreading activities of the enzymes.

4. Discussion

Using a combination of QMC-PCR, COLD-PCR, and Sanger sequencing, 10 non-recurrent variants were detected in the endonuclease domains of *POLE* and *POLD1* in 12% of CRC cases. The QMC-PCR demonstrated excellent performance in amplifying FFPE DNA, which facilitated effective mutation detection by HRM analyses [16]. The COLD-PCR, an established method for enriching minor mutant alleles, was also employed.[18] We have also previously utilized COLD-PCR followed by HRM analyses (COLD-HRM) to differentiate between germline and somatic mutations.[23]

First, the age of *POLE/POLD1*-mutant CRC patients was significantly lower than that of the wild-type patients, indicating a potential association between younger age and these mutations. This finding aligns with previous studies suggesting that *POLE* and *POLD1* mutations are more prevalent in younger CRC patients and may contribute to early-onset CRC. This observation aligns with prior research indicating a higher prevalence of POLE and *POLD1* mutations in younger CRC patients, implicating them in early-onset CRC.[24] Second, the

Table 3. Clinicopathological and molecular features of *POLE/POLD1*-mutated colorectal cancer

Features	*POLE/POLD1*-wild type	*POLE/POLD1*-mutant	*P*-value
Age	69.73 (±1.26) years	63.22 (±3.94) years	0.093
Gender			
Male	45	5	0.731
Female	28	4	
Anatomical sites			
Colon	50	8	0.271
Rectum	23	1	
Tumor size	4.78 (±0.23) cm	6.67 (±1.33) cm	0.199
Tumor grade			
Low grade	1	0	1.000
High grade	70	9	
Tumor stage			
T2 and earlier	10	0	0.592
T3 and later	62	9	
Nodal stage			
N0	32	3	0.725
N1	40	6	
TNM stage			
Stages 1 and 2	30	3	0.731
Stages 3 and 4	42	6	
Perineural invasion			
Absent	49	6	1.000
Present	21	3	
Vascular invasion			
Absent	31	1	0.080
Present	41	9	
Residual tumor			
Absent	67	9	1.000
Present	4	0	
Duke stage			
Stages A and B	32	3	0.724
Stages C and D	38	6	
Microsatellite status			
MSS	68	9	0.549
MSI	5	1	
Ploidy status			
Aneuploid	37	8	0.137
Diploid	29	2	

Abbreviations: MSI: Microsatellite instability; MSS: Microsatellite stable.

Table 4. ***In silico*** **prediction of the functional effect of the** ***POLE*** **and** ***POLD1*** **mutations**

Subject id	Amino acid substitution	Proven prediction		Sift prediction		Pon-P2 prediction	
		Score	Prediction (cutoff=-2.5)	Score	Prediction (Cutoff=0.05)	Probability for pathogenicity	Prediction
4	*POLE* L455Q	−5.36	Deleterious	0.001	Damaging	0.907	Pathogenic
32	*POLE* T278M	−5.23	Deleterious	0.001	Damaging	0.941	Pathogenic
38	*POLE* L432P	−6.46	Deleterious	0.000	Damaging	0.925	Pathogenic
40	*POLE* D414N	−4.61	Deleterious	0.000	Damaging	0.906	Pathogenic
44	*POLE* A374V	−1.95	Neutral	0.088	Tolerated	0.257	Neutral
63	*POLE* G433D	−1.06	Neutral	0.194	Tolerated	0.848	Pathogenic
64	*POLE* T457A	−2.27	Neutral	0.013	Damaging	0.523	Unknown
50	*POLD1* P404L	−8.65	Deleterious	0.005	Damaging	0.962	Pathogenic
52	*POLD1* A410V	−3.89	Deleterious	0.000	Damaging	0.954	Pathogenic

molecular characteristics of *POLE* and *POLD1* mutations play a crucial role in determining the microsatellite status and ploidy status of CRC patients. The majority of *POLE*/*POLD1*-mutant patients exhibited MSS and aneuploidy, highlighting distinct molecular profiles associated with these mutations. These findings underscore the importance of molecular subtyping in understanding the underlying biology of CRC and guiding personalized treatment approaches.

In our analysis of *POLE* and *POLD1* mutations in CRC patients, we identified several key mutations with significant implications for tumor biology and patient management. Notably, the exploration of these mutations has revealed both known variants reported in the literature and potentially novel variants that may contribute to the molecular diversity of CRC. The identification of mutations such as L455Q (c.1364 T>A), T278M (c.833 C>T), and L432P (c.1295 T>C) in POLE highlights the significance of alterations in the exonuclease domain, critical for maintaining DNA replication fidelity. These mutations, observed in CRC patients with MSS, underscore the importance of accurate DNA replication in preventing genomic instability and tumorigenesis.[1,25,26]

Similarly, mutations in *POLD1*, including P404L (c.1211 C>T) and A410V (c.1229 C>T), disrupt the proofreading function of the polymerase, leading to an accumulation of replication errors and genomic instability characteristic of CRC tumors.[27] These findings are consistent with previous studies implicating *POLE* and *POLD1* mutations in CRC predisposition and hereditary syndromes, highlighting their relevance in familial CRC cases and informing risk assessment and screening strategies for high-risk individuals.[3,28] Furthermore, the identification of these previously reported mutations in the *POLE* and *POLD1* genes emphasizes their relevance as biomarkers for patient stratification and personalized treatment approaches in CRC. By understanding the functional implications of these mutations, clinicians can better tailor therapeutic strategies to target specific molecular alterations in CRC patients.

Moreover, the functional consequences of *POLE* and *POLD1* mutations extend beyond CRC development, with implications for therapeutic interventions and clinical management. For instance, mutations such as L455Q and T278M in *POLE* may confer resistance to certain chemotherapeutic agents, necessitating alternative treatment approaches in CRC patients harboring these variants.[25] In addition, the identification of hypermutator phenotypes associated with specific *POLE* mutations underscores the potential for immunotherapy strategies in CRC patients with mismatch repair–deficient tumors, providing new avenues for personalized treatment approaches.[29,30]

Comparing our findings with previous studies reveals commonalities and insights into the functional consequences of *POLE* and *POLD1* mutations in CRC. For instance, the identification of the D414N mutation (c.1240 G>A) in *POLE*, similar to mutations reported in metastatic CRC tumors, highlights the role of these variants in tumor progression and metastasis.[26,31] In addition, the presence of truncating mutations such as S461* (c.1382 C>A) in *POLE* underscores the diverse spectrum of genetic alterations contributing to CRC pathogenesis, with implications for tumor aggressiveness and patient prognosis.[3]

5. Conclusion

Our study provides valuable insights into the role of *POLE* and *POLD1* mutations in CRC pathogenesis and clinical management. By elucidating the functional consequences of these mutations and their implications for tumor biology and therapeutic response, we advance our understanding of CRC heterogeneity and lay the groundwork for precision medicine approaches in CRC treatment.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization: Hersh Abdul Ham-Karim, Mohammad Ilyas
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Investigation: Hersh Abdul Ham-Karim, Narmeen Salih Ahmad
Methodology: Hersh Abdul Ham-Karim, Mohammad Ilyas
Writing-original draft: Hersh Abdul Ham-Karim, Mohammad Ilyas
Writing-review & editing: Narmeen Salih Ahmad, Mohammad Ilyas

Ethics approval and consent to participate

Access to tissues and ethics approval were granted by Nottingham Health Sciences Biobank, which has approval as an IRB from the North West – Greater Manchester Central Research Ethics Committee (REC reference: 15/NW/0685).

Consent for publication

Patient consent was not required for this manuscript, as no live tissues were utilized.

Availability of data

All datasets on which the conclusions of this paper rely have been presented in the main manuscript and the additional supporting files.

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Appendix

Table A1. The outer primer sequences

Gene/Exon	Forward	Reverse	Amplicon size
POLE/Exon 9	5'ATGGGGAGTTTAGAGCTTGG'3	5'CCATCCCAGGAGCTTACTTC'3	192 bp
POLE/Exon 10	5'GCAGCCTCTGACTTGTGC'3	5'CCGTTCTTCCCACAATACC'3	200 bp
POLE/Exon 11	5'GCAGACCTCTGACTGCTGTG'3	5'CCCTCCCTCTCAAATGCTG'3	167 bp
POLE/Exon 12	5'GGCATTAGAGCCTGACCTG'3	5'CACGGACAGCAGTGAGGAG'3	214 bp
POLE/Exon 13	5'CGGGCTGCATGTTAGAATC'3	5'AGCCGGGATGTGGCTTAC'3	205 bp
POLE/Exon 14	5'TTTGATGGCCCTGCTCTC'3	5'TCCATTCAGCTCCAGTGC'3	236 bp
POLD1/Exon 8/A	5' CTGGGAAATACGCCCTGAG'3	5' CGATATCGAAGCTGAGCAC'3	220 bp
POLD1/Exon 8/B	5' ACGTGCTGTGGTCTGACG'3	5' CCTTCATGCTTGCCATTCC'3	201 bp
POLD1/Exon 9	5' AGCCTCCCTGCTGTGTTG'3	5' GGAGCTGATGGCTCAGGAC'3	304 bp
POLD1/Exon 10	5' CACCAGGGTGACCCAATG'3	5' GAGGCACACAGAGCAAGATG'3	255 bp
POLD1/Exon 11	5' TGTGGTTGGTCTCAATCTCC'3	5' CCTTCCAAGGACAGGGACAC'3	263 bp
POLD1/Exon 12	5' GGGTGTGTCCCTGTCCTTG'3	5' CAGCAGGTCAAAGAGTGAGG'3	293 bp
POLD1/Exon 13/A	5' AGGCTACCTCACCCTGACC'3	5' GCCACGACTGAGCAGGTAG'3	247 bp
POLD1/Exon 13/A	5'ATGCCTACCTGCCACTGC'3	5'GGATGCCATTGCCTGGAG'3	215 bp

Note: Two sets of primers were used to amplify exon 8 and exon 13 of the POLD1 gene.

Table A2. The nested primer sequences

Gene/Exon	Forward	Reverse	Amplicon size size
POLE/Exon 9	5'TGCTTATTTTGTCCCCACAG'3	5'TACTTCCCAGAAGCCACCTG'3	153 bp
POLE/Exon 10	5'CCTCTGACTTGTGCTGATTGC'3	5'CCGGGTAGTTTCCCAAGTG'3	179 bp
POLE/Exon 11	5'CTGCTGTGACTTGGGTTCAG'3	5'CAAATGCTGCCCAGTTACTC'3	133 bp
POLE/Exon 12	'TGTCTGTCCTCTTTCCAACC'3	5'AGCCATGCTGCTCTGTGG'3	153 bp
POLE/Exon 13	5'TGGCTTCTGTTCTCATTCTCC'3	5'CGGGATGTGGCTTACGTG'3	179 bp
POLE/Exon 14	5'CTCTGGCGTTCTCTCCTCAG'3	5'GCCGACAGGACAGATAATGC'3	158 bp
POLD1/Exon 8/A	5'TGAGGCTGAAGGAGAAGGTG'3	5'CTTCCGGTGGGTGACTGAC'3	157 bp
POLD1/Exon 8/B	5'GCTGTGGTCTGACGTGGTC'3	5'GCTTGCCATTCCCTCCAC'3	190 bp
POLD1/Exon 9	5'GTGAGGGGCAGGAGTCAG'3	5'GCGTGGAGCGAGAGCTAC'3	228 bp
POLD1/Exon 10	5'AGGGTGACCCAATGTGCTC'3	5'GAGAGAAGCCTCCCACCTG'3	165 bp
POLD1/Exon 11	5'CACACCCTGCCTCTCCTC'3	5'CCAAGGACAGGGACACACC'3	200 bp
POLD1/Exon 12	5'GTCCTTGGAAGGCCACTG'3	5' GTCAGAGGTTGGGGTGAGAG'3	207 bp
POLD1/Exon 13/A	5'CCTTCTCCTGCTCCACCTC'3	5'CAGTGACCCTCGCCATCTC'3	189 bp
POLD1/Exon 13/B	5'TCATGGTGCTGGTGAACG'3	5'GACAAGTCTCGGCTACTGACC'3	130 bp

Note: Two sets of primers were used to amplify exon 8 and exon 13 of the POLD1 gene.

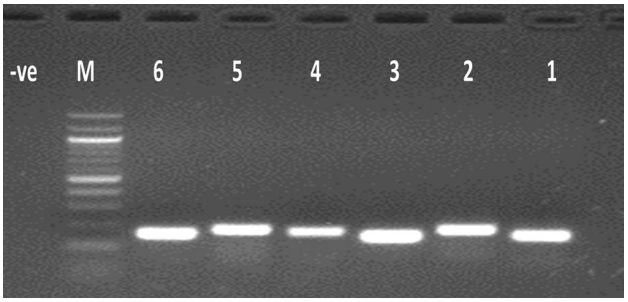


Figure A1. *POLE* inner primer specificity testing. Gel electrophoresis shows specific products generated by PCR using inner primers for all the *POLE* exons examined in this study. Lane 1: exon 9; lane 2: exon 10; lane 3: exon 11; lane 4: exon 12; lane 5: exon 13; lane 6: exon 14. Notes: M: DNA ladder; -ve: negative control.

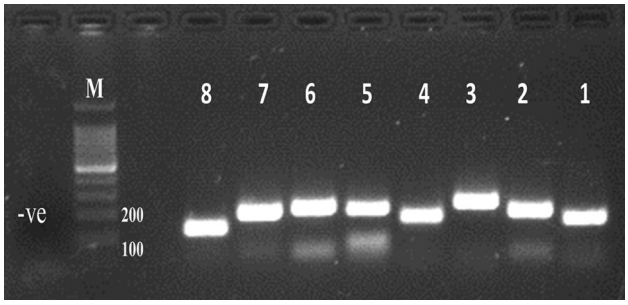


Figure A2. *POLD1* inner primer specificity testing. Gel electrophoresis showing specific products generated by PCR using inner primers for all the *POLD1* exons examined in this study. Lanes 1 and 2: exon 8; lane 3: exon 9; lane 4: exon 10; lane 5: exon 11; lane 6: exon 12; lanes 7 and 8: exon 13. Notes: M: DNA ladder; -ve: negative control.