High prevalence of unusual KRAS, NRAS, and BRAF mutations in POLE-hypermuted colorectal cancers

Loetitia Favre1,2, Justine Cohen1, Julien Calderaro1,2, Adrien Péciaux1, Cong-Trung Nguyen2, Rémi Bourgoin1, Laura Larnaudie1, Aurélie Dupuy2, Marie Ollier1, Emmanuëlle Lechapt1,2, Ivan Sloma2,3, Christophe Tournigand2,4, Benoit Rousseau4,5 and Anaïs Pujals1,2

1 Département de Pathologie, AP-HP, Centre Hospitalier Universitaire Henri Mondor, Créteil, France
2 INSERM, IMRB, Univ Paris Est Créteil, France
3 Département d’Hématologie Biologique, AP-HP, Centre Hospitalier Universitaire Henri Mondor, Créteil, France
4 Service d’Oncologie Médicale, AP-HP, Centre Hospitalier Universitaire Henri Mondor, Créteil, France
5 Mortimer B. Zuckerman Research Center, Memorial Sloan Kettering Cancer Center, New York, NY, USA

Keywords
colorectal cancers; immunotherapy; POLE; polymerase epsilon

Correspondence
A. Pujals, Département de Pathologie, AP-HP, UHC Henri Mondor, 1 rue Gustave Eiffel, 94010 Créteil, France
Tel: +33 1 49 81 27 47
E-mail: anais.pujals@aphp.fr

Benoit Rousseau and Anaïs Pujals contributed equally to this work

(Received 22 December 2021, revised 5 April 2022, accepted 25 May 2022, available online 14 July 2022)
doi:10.1002/1878-0261.13257

1. Introduction

DNA replication in the S phase of cell cycle involves multiple enzymes including DNA polymerases. These polymerases have activity in both DNA synthesis and DNA repair. Polymerase epsilon, encoded by the POLE gene, carries a proofreading (exonuclease) domain allowing error correction during replication ensuring a high-fidelity replication process. In tumors, POLE mutations affecting the exocluneasic domain result in a deficient DNA repair activity and a hyper/ultramutated cancer phenotype[1,2].

It has been recently reported that germline POLE mutations are risk factors for colorectal cancer (CRC)
and other tumor types, including endometrial cancer (EC) [3]. Somatic POLE mutations seem to be more frequent than germline mutations and are found in 5–10% of EC and in 3% of CRC [4–6]. Three main hotspots have been described in POLE-mutated EC and CRC (codons 286, 411, and 459) and other rare variants, all in the exonuclease domain, has also been described and associated with a hypermutated phenotype [7]. Interestingly, tumors with mutations in these hotspots are mostly microsatellite stable (MMR-P). The mean number of genomic mutations in tumors bearing a POLE pathogenic mutation appears to be 10-to 40-fold higher compared to a population of patients with MMR-P tumors. As a consequence, POLE exonuclease domain-mutated tumors define a new hypermutated non MMR-Deficient (MMR-D) subtype of cancer.

Hypermutated status is classically assessed by the tumor mutational burden (TMB), an exomic measure of the nonsynonymous mutations per megabase (mt/Mb), and defined by a TMB ≥ 10 mt/Mb. High TMB correlates with increased likelihood for a tumor of harboring immunogenic mutation-derived neoantigens and benefit derived from immunotherapy in specific tumor types [8]. While TMB has been recently approved in the United States as an agnostic biomarker to indicate immune checkpoint inhibitor [9], TMB assessment is neither available nor approved in most countries because of lack of randomized clinical trials. Recently, POLE mutations have been suggested to be an emergent biomarker for response to immunotherapy [10,11], underlining the need for dedicated screening strategies when TMB is not available in clinical practice.

We previously published a study investigating clinical and molecular profile of POLE-mutated CRC cancers based on available public data [12–14]. We showed that exonuclease domain POLE mutations prevalence was 2.3% in 967 CRC analyzed. The most frequent mutations were P286R/H, V411L (n = 3/22), and S459F (n = 4/22). The aim of this study was to confirm these observations using exploratory and validation cohorts and to improve POLE hypermutated CRC characterization based on clinico-molecular criteria.

2. Materials and methods

2.1. Sample selection and design

This study was designed to define clinical, histological, and molecular criteria for the screening of POLE-mutated patients applicable in clinical practice.

First, based on opensource databases (cbioportal.org), we generated an exploratory cohort to define the best clinico-molecular parameters associated with a POLE hotspot mutations.

Then, we prospectively sequenced, 420 samples of patients with CRC, corresponding to the validation dataset to evaluate whether the screening method would improve the proportion of hypermutated POLE-mutated MMR-P patients. Two hundred and twenty-two samples were allocated to the unselected cohort (unsupervised control screening) while 198 samples were allocated to the selected cohort (experimental screening) defined by the presence of noncodon 12 KRAS mutation, noncodon 600 BRAF mutation, or noncodon 12 NRAS mutation (Fig. 1).

All samples, had previously undergone molecular analysis in our Department of Pathology between 2015 and 2018 for determination of KRAS and NRAS status, in compliance with French regulations. The prerequisite for sample selection was the availability of residual archival extracted DNA to perform POLE PCR analysis. The following items were systematically recorded: age, sex, stage of the disease, location of the tumor, Mismatch Repair (MMR) status, KRAS, NRAS, BRAF, and PIK3CA mutational status.

2.2. Ethical approval

DNA was extracted from FFPE tissue blocks for medical diagnosis in compliance with French Regulations. The local ethics committee of Henri Mondor University Hospital gave its approval for this study (IRB No. 00011558; 2021–123). Experiments were undertaken with the understanding of each subject. A letter of nonobjection in connection with this study was sent to each patient. The study was performed in accordance with the Declaration of Helsinki.

2.3. DNA extraction

All DNA samples had been extracted as previously described [15–17], after macrodissection when necessary, from formalin-fixed paraffin-embedded (FFPE) tissue sections (usually 7 sections, 5-μm thick) using the Maxwell 16 FFPE Plus LEV DNA Purification Kit IVD (Promega, Charbonnières-les-Bains, France), according to the manufacturer’s instructions. The DNA was quantified using a Qubit fluorimeter in combination with the Qubit dsDNA HS Array Kit (ThermoFisher Scientific, Waltham, MA, USA).

2.4. High resolution melting PCR

POLE status was determined using high resolution melting (HRM) performed with a LightCycler® 480
Roche, Basel, Switzerland) using specific primers (Table S1) for all cases. All samples were tested in duplicate. One positive mutated DNA sample and two wild-type DNA samples were included as controls in each run. HRM experiments have already been described previously [15]. The final volume of the PCR reaction was 20 μL, containing 10 μL of LightCycler 480 HRM MasterMix (Roche), 3 mM of MgCl₂, 0.2 μM each primer, 0.5 U of Uracil-NGlycosylase, and 20 ng of DNA, as measured by fluorimetry. DNA samples were treated with uracil glycosylase before amplification to avoid artifacts due to cytosine deamination. The cycling protocol was performed as follows: incubation at 37 °C for 10 min, denaturation at 95 °C for 10 min, 45 cycles of amplification (10 s at 95 °C, 15 s at 55 °C, and 30 s at 72 °C), followed by a melting curve (denaturation at 95 °C, hybridization at 40 °C, and melting from 70 to 95 °C). Melting curves from the samples were automatically normalized and analyzed with LIGHTCYCLER 480 software (Roche).

2.5. Next generation sequencing

Patients with mutated profiles identified by HRM PCR were then analyzed using Next Generation Sequencing (NGS) as previously described [15,16]. For NGS, 10 ng of DNA (as measured by fluorimetry) was amplified using the Ion AmpliSeq™ OST+ V2 panel (ThermoFisher Scientific), which is a multiplex PCR-based library-preparation method by which many regions (70–150 bp) that encompass many mutational hotspots including POLE codons 286, 411, and 459 are amplified. Amplicons were then digested, barcoded, and amplified by using the Ion Oncomine™ Solid Tumor DNA Kit and Ion Select Barcode Adapter Kit (ThermoFisher Scientific), according to the manufacturer’s instructions. After DNA quantification, 25 pm of each library was multiplexed and clonally amplified on Ion Sphere particles (ISP) by emulsion PCR performed on Ion Chef (ThermoFisher Scientific), according to the manufacturer’s instructions. The ISP templates were loaded onto an Ion-520 chip and sequenced on a S5 sequencer with the Ion 510™ & Ion 520™ & Ion 530™ Kit–Chef, according to the manufacturer’s instructions. Run performance was assessed and data analyzed with the TENTURE SUITE Software v.5.10.0 (ThermoFisher Scientific). Single-nucleotide variants and small indels were detected using the Variant Caller plug-in version 5.10.0.18 with low stringency settings (threshold: 2%). The Integrative Genomics Viewer (IGV v 5.01; Broad Institute, Cambridge, MA, USA) was used for visual inspection of the aligned reads.

Tumor mutational burden (TMB) was assessed for patients harboring a POLE mutation using FoundationOne® CDX (Roche). FiCDx comprehensive genomic profiling (CGP) has been performed based on the
method described by Frampton et al. Tumor mutational burden was evaluated using FoundationOne® method according to Szustakowski et al.\[18\]. Tumors were considered ultra-mutated if they contained more than 100 mutations/Megabases (mt/M) hypermutated if they contained between 10 and 100 mt/Mb and with low TMB if they contained less than 10 mt/Mb.

2.6. Immunochemistry

IHC analysis was performed in MMR-P \((n = 20)\), MMR-D \((n = 20)\), and POLE-mutated \((n = 11)\) tissue sections. IHC was carried out on FFPE tissue sections, as previously described \[17\], using antibodies against MLH1 (mouse mAb, clone G168.728; Microm Mitech, Brignais, France), PMS2 (mouse mAb, clone A16-4, 1 : 100; BD Pharmingen, Le Pont de Claix, France), MSH2 (mouse mAb, clone FE11, 1 : 100; Biocare Medical, Pacheco, CA, USA), MSH6 (rabbit mAb, clone EP49, 1 : 100; CliniSciences, Nanterre, France), CD3 (mouse mAb, clone F7.2.38 1 : 50; Agilent Dako, Les Ulis, France), CD8 (mouse mAb, clone C8/144B, 1 : 200; Agilent Dako), CD20 (mouse mAb, clone L26 1 : 500; Agilent Dako), and PDL1 (rabbit mAb, clone QR1, 1 : 100; Diagomics/quartett). IHC was performed on a BOND III or a BOND-MAX (Leica, Nanterre, France) automated stainer platform. The expression of the four MMR proteins defined a stable phenotype (MMR-P). Staining pattern consisted in nuclear staining within tumor cells with infiltrating lymphocytes, as positive internal controls. The loss of one or more proteins characterized by a total absence of nuclear staining within tumor cells with a positive labeling of nontumor cells, defined microsatellite unstable phenotype (MMR-D). PD-L1 expression was evaluated on tumor cells and immune cells by a pathologist. Membranous cell staining was quantified to give a percentage of positive PD-L1 cells. Immunolabeling for CD3, CD8, and CD20 were evaluated and quantified using QUPATH software (version 0.2.0, University of Edinburgh, Edinburgh, UK).

2.7. Statistics and public database

The primary endpoint of this study was to identify criteria, using an exploratory cohort to screen patients for \textit{POLE} hotspot mutations. Using cBioPortal, four Table 1. Characteristics of POLE-mutated patients in opensource data.

| POLE-mutated cases (%) | 20 (0.8) |
|------------------------|---------|
| Mean of age (year) [Range] | 59 [24–86] |
| Location (%) | 10 (50) |
| Right colon | 4 (20) |
| Left colon | 4 (20) |
| Unknown | 2 (10) |
| Sex (%) | 13 (65) |
| M | 5 (25) |
| F | 2 (10) |
| POLE mutation (%) | 9 (45) |
| Codon 286 | 6 (30) |
| Codon 459 | 5 (25) |
| KRAS mutation (%) | 7 (35) |
| WT | 0 (0) |
| Noncodon 12 mutation | 13 (65) |
| NRAS mutation (%) | 16 (80) |
| WT | 4 (20) |
| Noncodon 12 mutation | 0 (0) |
| BRAF mutation (%) | 12 (60) |
| WT | 8 (40) |
| Noncodon 600 mutation | 9 (45) |
| PIK3CA mutation (%) | 11 (55) |
| WT | 13 (65) |
| Noncodon 600 mutation | 1 (5) |
| Unknown | 6 (30) |

Statistical analysis was performed using R software (3.2.2, R Foundation for Statistical Computing, Vienna, Austria). Relationship between qualitative variables were assessed using Chi square tests with Monte Carlo resampling method for multiple hypothesis testing. For IHC, statistical analysis was performed using GRAPHPAD PRISM (San Diego, CA, USA). Mann–Whitney two-tailed test was used.

3. Results

3.1. Molecular analysis of POLE-mutated tumors identified in cBioPortal platform

The first objective of our study was to identify criteria, using an exploratory cohort to screen patients for \textit{POLE} hotspot mutations. Using cBioPortal, four
CRC cohorts published with opensource sequencing data were analyzed (Table 1): the TCGA provisional (n = 640) [6]; MSKCC (n = 1134) [19]; DFCI (n = 619) [20]; and Genentech (n = 72) [21]. Review of available individual genomic data of CRC samples in these four cohorts revealed 20 samples (0.8%) with mutations in the three hotspots described in POLE exonuclease domain (codons 86–460). The mean age at diagnosis was 59 (Table 1). Patients with POLE mutation were more frequently men (65% vs 25% female and 10% unknown) and tumors were mostly located in right colon (50%). As expected, most of POLE mutations affected codon 286 (P286R/H; 45%), codon 459 (S459F; 30%), and codon 411 (V411L; 25%). Interestingly, these POLE mutations were predominantly associated with KRAS, NRAS, BRAF, and/or PIK3CA mutations, commonly found in colorectal cancers. But unlike classical CRC, POLE-mutated tumors were strongly associated with unusual mutations, which exhibit rare prevalence in these genes: 65% harbored noncodon 12 KRAS mutations (G13D; V14I; D57N; E98*; K117N; A146T; K147T/E or K170Q); 20% harbored noncodon 12 NRAS mutation (Q61R; E132K; R167*); 40% harbored a non-V600E BRAF mutation (F294L; Y633C; L312P; S602Y; Q356K; R354*; S102Y; L567V; R389C; F247L); 55% harbored a PIK3CA mutation. In these series, 55% of the cases presented multiple concomitant unusual mutations. Regarding MMR status, 65% were MMR-P, 5% were MMR-D, and 30% were unknown. Altogether, these results suggest that hotspot POLE-mutated tumors in CRC have molecular characteristics, such as MMR-P status and noncodon 12 mutations of KRAS/NRAS and noncodon 600 BRAF mutation (P = 0.02).

3.2. Screening for POLE-mutated CRC in the exonuclease domain in a cohort of nonselected or cases selected for unusual mutation of KRAS, NRAS, and BRAF

Based on these findings, we prospectively assessed if selecting cases with noncodon 12 KRAS/NRAS mutations and noncodon 600 BRAF mutation would increase the prevalence of POLE-mutated MMR-P CRC in the exonuclease domain compared with an unselected population. Between June 2013 and September 2018, a total of 420 patients, screened for RAS status in our lab, were included in this study and divided into two groups: a nonselected control cohort (n = 222) of patients and a selected experimental cohort (n = 198) specifically selected on the presence of noncodon 12 KRAS/NRAS mutations and noncodon 600 BRAF mutation associated with a MMR-P status. Patient characteristics of these two groups are shown in Table 2. The mean age of these patients was 68 for both cohorts, ranging from 27 to 96 for the nonselected cohort and from 27 to 94 in the selected cohort. Male to female sex ratio was 1.3 for both cohorts. All stages CRC were included in the study. At the molecular level, tumors were predominantly MMR-P in both cohort (91% and 95% for the nonselected and selected cohort, respectively). The nonselected cohort contained mostly cases with a KRAS/NRAS/BRAF/PIK3CA

| Table 2. Clinical and molecular characteristics of patients included in the two cohorts. |
|-----------------------------------------------|-----------------|-----------------|
|                                               | Nonselected cohort | Selected cohort |
| **n**                                         | 222              | 198             |
| **Age (years)**                               | 68 (27–96)       | 68 (27–94)      |
| **Sex**                                       |                  |                 |
| Male                                          | 126 (57%)        | 112 (57%)       |
| Female                                        | 96 (43%)         | 86 (43%)        |
| **Stage**                                     |                  |                 |
| I                                             | 5 (2%)           | 4 (2%)          |
| II                                            | 45 (20%)         | 29 (15%)        |
| III                                           | 69 (31%)         | 74 (37%)        |
| IV                                            | 42 (19%)         | 39 (20%)        |
| Unknown                                       | 61 (28%)         | 52 (26%)        |
| **Location**                                  |                  |                 |
| Right                                         | 77 (35%)         | 69 (35%)        |
| Left                                          | 76 (34%)         | 57 (29%)        |
| Rectum                                        | 48 (22%)         | 46 (23%)        |
| Unknown                                       | 21 (9%)          | 26 (13%)        |
| **MMR status**                                |                  |                 |
| MMR-P                                         | 202 (91%)        | 188 (95%)       |
| MMR-D                                         | 10 (4.5%)        | 0 (0%)          |
| Unknown                                       | 10 (4.5%)        | 10 (5%)         |
| **KRAS status**                               |                  |                 |
| Wild-type                                     | 120 (54%)        | 26 (13%)        |
| Typical mutation (codon 12)                   | 63 (28%)         | 0 (0%)          |
| Atypical mutation                             | 33 (15%)         | 172 (87%)       |
| Unknown                                       | 6 (3%)           | 0 (0%)          |
| **BRAF status**                               |                  |                 |
| Wild-type                                     | 122 (55%)        | 155 (78%)       |
| Typical mutation (codon 600)                  | 19 (9%)          | 0 (0%)          |
| Atypical mutation                             | 10 (4%)          | 29 (15%)        |
| Unknown                                       | 71 (32%)         | 14 (7%)         |
| **NRAS status**                               |                  |                 |
| Wild-type                                     | 139 (63%)        | 175 (88%)       |
| Mutation                                      | 13 (6%)          | 8 (4%)          |
| Unknown                                       | 70 (31%)         | 15 (7.5%)       |
| **PIK3CA status**                             |                  |                 |
| Wild-type                                     | 126 (57%)        | 155 (78%)       |
| Mutation                                      | 23 (10%)         | 29 (15%)        |
| Unknown                                       | 73 (33%)         | 14 (7%)         |
| **POLE status**                               |                  |                 |
| Wild-type                                     | 221 (99.5%)      | 188 (95%)       |
| Mutation (exonuclease domain)                 | 1 (0.5%)         | 10 (5%)         |
wild-type status or with typical mutations of these genes (codon 12 mutation for KRAS, codon 12 mutation for NRAS and codon 600 mutation for BRAF).

As defined, the selected cohort had a high rate of tumors with noncodon 12 KRAS mutation (87% vs 15% in nonselected cohort) and noncodon 600 BRAF mutation (15% vs 4% in the nonselected cohort).

We identified 10 tumors out of 198 (5%) carrying POLE mutation in the exonuclease domain in the selected cohort whereas only one tumor out of 222 (0.5%) was mutated in the nonselected cohort (Chi-square test, \( P = 0.0032 \)). Of note, the latter mutated sample was observed in a MMR-D tumor while the 10 other mutated tumors were all MMR-P. These results highlight the benefits of screening preferentially CRC samples with KRAS/BRAF/NRAS unusual mutation(s) to improve the identification of POLE-mutated patients in the exonuclease domain. The molecular screening criteria lead to an enrichment by 10-fold in the prevalence of POLE mutations.

### 3.3. Molecular characteristics of the POLE-mutated tumors identified during the study

The characteristics of the 11 cases of POLE-mutated tumors in the exonuclease domain are presented in Table 3. Four POLE mutations were found on codon 286, two on codon 411, one on codon 425, one on codon 459, one on codon 461, and two on codon 464. One of these mutations on codon 464 was a silent mutation (V464V, case number 11) and corresponds to a MMR-D case identified in the nonselected cohort. The median age of these patients was 54.2, ranging from 31 to 73 years old. They were predominantly male (\( n = 9, 82\% \)) and CRC were mostly left-sided (\( n = 6, 55\% \)). Except case number 11, all tumors were MMR-P.

Among these 11 tumors, 9 (82%) carried a non-codon 12 KRAS mutation (G13D; A59T; N116H; K117N; A146V/T), associated or not with BRAF or PIK3CA mutations. Two tumors (18%) carried a non-codon 600 BRAF mutation (D454V or D594G). One tumor (9%) carried a double NRAS mutation (Q61R and T58A) and one tumor (9%) carried a PIK3CA mutation (E542K). In this series, POLE mutation was associated with KRAS mutation on codon 146. The mutational burden score of these tumors was assessed with FoundationOne® CDX (Roche). Among the 11 POLE-mutated cases, 6 were ultramutated \( \geq 100 \) mutations/Megabases (mt/Mb, cases number 1, 2, 5, 6, 7, 8) 3 were hypermutated \( \geq 10 \) and \( < 100 \) mt/Mb (cases number 9, 10, 11) and 2 had low TMB \( < 10 \) mt/Mb (cases number 3 and 4).

| Table 3. molecular characteristics of POLE-mutated patient. |
|----------------------------------------------------------|
| **n** | 11 |
| **Age (years)** | 54.2 (31–73) |
| **Sex** | |
| Male | 9 (82%) |
| Female | 2 (18%) |
| **Location** | |
| Right | 3 (27%) |
| Left | 6 (55%) |
| Rectum | 1 (9%) |
| Unknown | 1 (9%) |
| **MMR status** | |
| MMR-P | 10 (91%) |
| MMR-D | 1 (9%) |
| **KRAS status** | |
| Wild-type | 2 (18%) |
| Typical mutation (codon 12) | 0 (0%) |
| Atypical mutation | 9 (82%) |
| **BRAF status** | |
| Wild-type | 9 (82%) |
| Typical mutation (codon 600) | 0 (0%) |
| Atypical mutation | 2 (18%) |
| **NRAS status** | |
| Wild-type | 2 (18%) |
| Mutation | 1 (9%) |
| **PIK3CA status** | |
| Wild type | 10 (91%) |
| Mutation | 1 (9%) |

### 3.4. Evaluation of the infiltration of POLE-mutated tumors by immune cells

Several studies have shown that MMR-D CRC have higher rate of CD8+ tumor-infiltrating lymphocytes (TILs) than MMR-P tumors [22–24]. We therefore assessed if POLE-mutated CRC tumors had an immune profile closer to MMR-D than other MMR-P tumors. We performed IHC to compare the density of CD3+, CD8+, and CD20+ TILs, between MMR-P (\( n = 20 \)), MMR-D (\( n = 20 \)), and POLE-mutated (\( n = 11 \)) -CRC. Our results show that MMR-P tumors have the lowest densities of CD3+ CD8+ and CD20+ TILs as compared to MMR-D and POLE-mutated tumors (Fig. 2). No significant difference was observed between the three groups for CD3+ TILs. However, CD8+ TILs were significantly higher in POLE-mutated CRC (P = 0.0073) and in MMR-D tumors (P = 0.0493) than in MMR-P tumors. Similarly, CD20+ TILs were higher in POLE-mutated tumors than in MMR-P tumors (P = 0.0310; Fig. 2). Representative immunostaining for MMR-P, MMR-D, and POLE-mutated CRC are illustrated in Fig. 3. Immunohistochemistry staining was then performed to evaluate PD-L1 expression in POLE-mutated tumors. Results showed that
tumor cells were most frequently PD-L1 negative (n = 8) or with low staining (tumor cells less than 10%, n = 2; Table 4) suggesting that POLE-mutated tumors are not associated with PD-L1 expression. Representative PD-L1 staining is shown in Fig. 3.

Altogether, these data indicate that POLE-mutated CRC had an immune profile closer to MMR-D than MMR-P tumors, suggesting they could exert an antitumor activity in POLE-mutated CRC so as in MMR-D CRC, independently of PD-L1 expression.
Table 4. Clinicopathological characteristics of edPOLE-mutated patients.

| Sex | Age | Localization | Stage | MMR status | Mutations | PolE mutation | Mutational burden score | IHC PDL1 tumor cells | IHC PDL1 immune cells |
|-----|-----|--------------|-------|------------|-----------|---------------|------------------------|----------------------|----------------------|
| M 47 | Rectum | NP | MMR-P | KRAS N116H; BRAF D454V | p.(Ser461Thr) | 226.95 | <1% | 40% |
| M 63 | Left | T4bN0 | MMR-P | KRAS A95T; BRAF D594G | p.(Pro286Arg) | 189.13 | 0% | 0% |
| F 73 | Sigmoid | NP | MMR-P | NRAS G13D | p.(Val411Leu) | 142.48 | 0% | 0% |
| M 64 | Unknown | M1 (liver) | MMR-P | KRAS K117N | p.(Val411Leu) | 417.34 | 20% | 5% |
| M 55 | Left | NP | MMR-P | KRAS A146T; POLE | p.(Val464Ala) | 166 | 0% | 0% |
| F 65 | Right | NP | MMR-P | KRAS A146V; POLE | p.(Val464Ala) | 142.48 | 0% | 0% |
| M 36 | Left | T4bN0M1 | MMR-P | KRAS A146T; POLE | p.(Val464Ala) | 218.13 | 5% | 5% |
| M 42 | Right | NP | MMR-P | KRAS A146T; POLE | p.(Val464Ala) | 78.17 | 10% | 10% |
| M 61 | Sigmoid | NP | MMR-P | KRAS G13D | p.(Ser459Phe) | 49.17 | 0% | 0% |
| M 59 | Right | T4aN2b | MMR-D | KRAS A59T; POLE | PIK3CA E542K | 56.74 | <1% | 0% |

4. Discussion

Exonucleasic domain POLE mutations occur in 1–2% of MMR-P CRC responsible for hypermutated phenotype and are emergent predictive biomarkers for response to immune checkpoint inhibitors. To our knowledge, this is the first study which demonstrate that edPOLE-mutated tumors are associated with high prevalence of noncodon 12 KRAS/NRAS mutations and noncodon 600 BRAF mutation. Furthermore, our preliminary data show that this observation is not found in other types of cancer and seems specific to CRC (data not shown). Restricting the edPOLE mutation screening to patients with tumors harboring these unusual mutations in KRAS, NRAS, and/or BRAF genes lead to an enrichment of the prevalence of edPOLE mutations up to 5% while in an unselected population the prevalence was only 0.5%. Clinically, edPOLE tumors were mostly observed in men with left-sided CRC, with a noncodon 12 KRAS mutation and hyper/ultramutated MMR-P. Microscopically, edPOLE-mutated tumors displayed high CD8+ TILs infiltration and were not associated with high expression of PDL-1 confirming previous observations [25,26]. Interestingly, a study performed by Domingo et al. [26] on 6517 colorectal cancers, showed 66 POLE-mutated tumors. In this cohort, only one case of POLE-mutated tumors was associated with KRAS mutation. Nevertheless, only KRAS exon 2 (codons 12 and 13) and BRAF codon 600 were analyzed in this study. Clinical observations are consistent with ours: POLE-mutated tumors were observed in young men but predominantly right-sided, whereas only 3 out of 11 POLE-mutated tumors were right-sided in our study. Another study performed on stage II CRC showed a higher percentage of edPOLE-mutated patients (3.1%) than in the four CRC cohorts published (Table 1) and clinicopathological characteristics are also identical to those mentioned above [27].

In our study, the method used to detect these mutations is based on a qualitative, rapid, and low-cost PCR. Identified POLE-mutated tumors need to be further sequenced to determine the exact mutation and its pathogenicity. As TMB is not an approved biomarker worldwide and because its assessment requires large sequencing panels or whole exome sequencing, POLE-targeted sequencing seems a seducing alternative when TMB is not available. Our study shows that the presence of a noncodon 12 KRAS or NRAS mutation should lead clinicians and biologists to look for the presence of POLE mutation. Moreover, recent reports also suggest that the benefit derived from immunotherapy in high TMB CRCs is limited to patients with tumors displaying DNA repair impairment such as in MMR-D and POLE proofreading deficiency. These data highlight the need to assess the underlying cause of high TMB in CRC to offer immunotherapy, making POLE assessment necessary for MMR-P tumors. However, our study shows that POLE exonucleasic domain mutations are not always associated with a high TMB. In our series, one patient was MMR-D, with a silent edPOLE mutation V464V. ClinVar predicts this variant as likely benign and might have low impact on Polymerase Epsilon function. In this case, high TMB was probably due to MMR-D phenotype instead of edPOLE mutation. Further studies are thus needed to assess which nonhotspot POLE mutations are pathogenic and driver in order to identify those who are correlated with a good response to immunotherapy.

First results of the program AcSé Nivolumab including 16 patients with MMR-P POLE-mutated tumors showed an overall response rate of 50% in
patients tumors harboring pathogenic exonucleasic domain mutations, all in advanced CRC. Conversely, no response was observed for the patients carrying non-pathogenic mutations. Comparing patients harboring nonpathogenic and pathogenic/unknown significance variants, a survival benefit was also observed (mOS = 5.3 months vs not reached, \(P\)-value = 0.003) [28]. Furthermore, several case reports describe immune checkpoint inhibitor efficiency in POLE-mutated CRC patients, regardless of the expression of PDL-1 [29,30]. As patients with edPOLE-mutated tumors seems to be extreme responders to immune checkpoint blockade, early identification of these patients in localized or advanced setting could allow clinicians to offer early immunotherapy-based strategies to avoid chemo/radiotherapy and improve clinical outcomes.

Finally, this study raises questions about the role of unusual mutations in the carcinogenesis of edPOLE-mutated tumors. Poulin et al. [31] demonstrated that there are different biochemical properties of KRAS mutations. KRAS G12D would activate the MAPK pathway more strongly than the KRAS A146T. In addition, global proteomic analysis revealed that KRAS A146T clustered more closely with KRAS WT than with KRAS G12D. Other data reinforce the hypothesis that not all KRAS mutations are equivalent. For instance, patients with KRAS G13D mutation would have a better outcome compared to patients mutated on codon 12 after cetuximab treatment [32]. Nevertheless, these data remain controversial and no clinical trial has demonstrated cetuximab benefit for patients with KRAS G13D [33–35].

In this context, in contrast to typical KRAS-mutated tumors, the initiating event of carcinogenesis could be the POLE mutation, as it has been shown by Temko et al. [36]. By occurring early, replication errors are no longer repaired and accumulate in the cells, resulting in a large number of neoantigens and a high TMB.

5. Conclusion

To conclude, this study improves our molecular understanding of POLE-mutated tumors and should encourage the search for POLE mutations in tumors with an atypical KRAS mutation, especially in young men with an MSS phenotype. Nevertheless, more work is needed to understand why high prevalence of unusual mutations in the RAS and BRAF genes are observed in these patients.

Acknowledgements

The authors thank Roche and Foundation Medicine Inc for the analysis of TMB. The authors thank the molecular biology technicians for their technical assistance. The author(s) received no specific funding for this work.

Conflict of interest

The authors declare no conflict of interest.

Author contribution

LF, JCo, AP, RB, and LL performed experiments. LF, JCo, AD, and C-TN analyzed immunolabeling. AP, BR, and CT designed the study. AD and MO participated in the study conception. LF wrote draft manuscript. LF, JCo, JCa, RB, LL, MO, and AP participated in sample selection. AD, JCa, EL, IS, CT, BR, and AP revised draft manuscript. All authors read and approved the final manuscript.

Data availability statement

All data generated or analyzed during this study are included in this published article.

Peer Review

The peer review history for this article is available at https://publons.com/publon/10.1002/1878-0261.13257.

References

1 Heitzer E, Tomlinson I. Replicative DNA polymerase mutations in cancer. *Curr Opin Genet Dev*. 2014;24:107–13.
2 Rayner E, van Gool IC, Palles C, Kearsey SE, Bosse T, Tomlinson I, et al. A panoply of errors: polymerase proofreading domain mutations in cancer. *Nat Rev Cancer*. 2016;16(2):71–81.
3 Bellido F, Pineda M, Aiza G, Valdés-Mas R, Navarro M, Puente DA, et al. POLE and POLD1 mutations in 529 kindred with familial colorectal cancer and/or polyposis: review of reported cases and recommendations for genetic testing and surveillance. *Genet Med*. 2016;18(4):325–32.
4 Travaglini A, Raffone A, Gencarelli A, Mollo A, Guida M, Insabato L, et al. TCGA classification of endometrial cancer: the place of carcinosarcoma. *Pathol Oncol Res*. 2020;26(4):2067–73.
5 Palles C, Cazier J-B, Howarth KM, Domingo E, Jones AM, Broderick P, et al. Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. *Nat Genet*. 2013;45(2):136–44.
Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012;487(7407):330–7.

Campbell BB, Light N, Fabrizio D, Zatzman M, Fuligni F, de Borja R, et al. Comprehensive analysis of hypermutation in human cancer. *Cell*. 2017;171(5):1042–56.e10.

Rousseau B, Foote MB, Maron SB, Diplas BH, Lu S, Argièles G, et al. The spectrum of benefit from checkpoint blockade in hypermutated tumors. *N Engl J Med*. 2021;384(12):1168–70.

Marabelle A, Fakih M, Lopez J, Shah M, Shapira-Frommer R, Nakagawa K, et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol*. 2020;21(10):1353–65.

Rousseau B, Vidal J, Diaz LA. Evaluation of POLE/POLD1 variants as potential biomarkers for immune checkpoint inhibitor treatment outcomes. *JAMA Oncol*. 2020;6(4):589–90.

Wang F, Zhao Q, Wang YN, Jin Y, He MM, Liu ZX, et al. Evaluation of POLE and POLD1 mutations as biomarkers for immunotherapy outcomes across multiple cancer types. *JAMA Oncol*. 2019;5:1304–6.

Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6(269):p11.

Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2(5):401–4.

Bourdais R, Rousseau B, Pujals A, Boussion H, Joly C, Guillemín A, et al. Polymerase proofreading domain mutations: new opportunities for immunotherapy in hypermutated colorectal cancer beyond MMR deficiency. *Crit Rev Oncol Hematol*. 2017;113:242–8.

Guyard A, Boyez A, Pujals A, Robe C, Tran van Nhieu J, Allory Y, et al. DNA degrades during storage in formalin-fixed and paraffin-embedded tissue blocks. *Virchows Arch*. 2017;471(4):491–500.

Bocciairelli C, Cohen J, Pelletier R, Tran van Nhieu J, Derman J, Favre L, et al. Evaluation of the Idylla system to detect the EGFR. *Pathol Res Pract*. 2020;216(1):152773.

Pécriaux A, Favre L, Calderaro J, Charpy C, Derman J, Pujals A. Detection of microsatellite instability in a panel of solid tumours with the Idylla MSI Test using extracted DNA. *J Clin Pathol*. 2021;74(1):36–42.

Sztukowski JD, Green G, Geese WJ, Zerba K, Chang H. Abstract 5528: Evaluation of tumor mutation burden as a biomarker for immune checkpoint inhibitor efficacy: a calibration study of whole exome sequencing with FoundationOne®. *Cancer Res*. 2018;78:5528.

Jaeger R, Chatila WK, Lipsyc MD, Hechtman JF, Crecer A, Sanchez-Vega F, et al. Clinical sequencing defines the genomic landscape of metastatic colorectal cancer. *Cancer Cell*. 2018;33(1):125–36.e3.

Giannakis M, Mu XJ, Shukla SA, Qian ZR, Cohen O, Nishihara R, et al. Genomic correlates of immune-cell infiltrates in colorectal carcinoma. *Cell Rep*. 2016;15(4):857–65.

Seshagiri S, Stawiski EW, Durinck S, Modrusan Z, Storm EE, Conboy CB, et al. Recurrent R-spondin fusions in colon cancer. *Nature*. 2012;488(7413):660–4.

Dolcetti R, Viel A, Doglioni C, Russo A, Guidoboni M, Capozzi E, et al. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol*. 1999;154(6):1805–13.

Phillips SM, Banerjea A, Feakins R, Li SR, Bustin SA, Dorudi S. Tumour-infiltrating lymphocytes in colorectal cancer with microsatellite instability are activated and cytotoxic. *Br J Surg*. 2004;91(4):469–75.

Scarpa M, Ruffolo C, Canal F, Scarpa M, Busato S, Erroi F, et al. Mismatch repair gene defects in sporadic colorectal cancer enhance immune surveillance. *OncoTarget*. 2015;6(41):43472–82.

Forgó E, Gomez AJ, Steiner D, Zehnder J, Longacre TA. Morphological, immunophenotypical and molecular features of hypermutation in colorectal carcinomas with mutations in DNA polymerase ε (POLE). *Histopathology*. 2020;76(3):366–74.

Domingo E, Freeman-Mills L, Rayner E, Glaire M, Briggs S, Vermeulen L, et al. Somatic POLE proofreading domain mutation, immune response, and prognosis in colorectal cancer: a retrospective, pooled biomarker study. *Lancet Gastroenterol Hepatol*. 2016;1(3):207–16.

Mo S, Ma X, Li Y, Zhang L, Hou T, Han-Zhang H, et al. Somatic POLE exonuclease domain mutations elicit enhanced intratumoral immune responses in stage II colorectal cancer. *J Immunother Cancer*. 2020;8(2):e00881.

Rousseau BJ, Bieche I, Pasmant E, Simmet V, Hamzaoui N, Masliah-Planchon J, et al. 526O- High activity of nivolumab in patients with pathogenic exonuclease domain POLE (edPOLE) mutated Mismatch Repair proficient (MMRp) advanced tumours. *Ann Oncol*. 2020;31:S463.

Gong J, Wang C, Lee PP, Chu P, Fakih M. Response to PD-1 blockade in microsatellite stable metastatic colorectal cancer harboring a POLE mutation. *J Natl Compr Canc Netw*. 2017;15(2):142–7.

Silberman R, Steiner DF, Lo AA, Gomez A, Zehnder JL, Chu G, et al. Complete and prolonged response to
immune checkpoint blockade in POLE-mutated colorectal cancer. *JCO Precis Oncol*. 2019;3:1–5.

31 Poulin EJ, Bera AK, Lu J, Lin YJ, Strasser SD, Paulo JA, et al. Tissue-specific oncogenic activity of KRAS. *Cancer Discov*. 2019;9(6):738–55.

32 De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, et al. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA*. 2010;304(16):1812–20.

33 Gajate P, Sastre J, Bando I, Alonso T, Cillero L, Sanz J, et al. Influence of KRAS p.G13D mutation in patients with metastatic colorectal cancer treated with cetuximab. *Clin Colorectal Cancer*. 2012;11(4):291–6.

34 Segelov E, Thavaneswaran S, Waring PM, Desai J, Robledo KP, Gebski VJ, et al. Response to cetuximab with or without irinotecan in patients with refractory metastatic colorectal cancer harboring the KRAS G13D mutation: Australasian Gastro-Intestinal Trials Group ICECREAM Study. *J Clin Oncol*. 2016;34(19):2258–64.

35 Schirripa M, Loupakis F, Lonardi S, Cremolini C, Bergamo F, Zagonel V, et al. Phase II study of single-agent cetuximab in KRAS G13D mutant metastatic colorectal cancer. *Ann Oncol*. 2015;26(12):2503.

36 Temko D, van Gool IC, Rayner E, Glaire M, Makino S, Brown M, et al. Somatic POLE exonuclease domain mutations are early events in sporadic endometrial and colorectal carcinogenesis, determining driver mutational landscape, clonal neoantigen burden and immune response. *J Pathol*. 2018;245(3):283–96.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Primers used for POLE HRM PCR.