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ARTICLE

High mutational burden in colorectal carcinomas with monoallelic *POLE* mutations: absence of allelic loss and gene promoter methylation

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Abstract

Hypermutator-type colorectal carcinomas are microsatellite-stable and have point mutations of the exonuclease domain of the DNA polymerase ε or δ genes (*POLE* and *POLD1*, respectively), and an ultrahigh tumor mutational burden (TMB). These tumors may be associated with enhanced antitumor immunity and preferentially afflict younger patients, but this notion awaits validation by accrual of further cases for detailed correlative phenotypic and molecular study. We performed *POLE* and *POLD1* exonuclease domain Sanger sequencing of 271 unselected colorectal carcinomas. We identified two microsatellite-stable tumors with somatic *POLE* p.P286R variants, both with ultrahigh TMBs as demonstrated by whole exome sequencing. A *POLE* p.V411L was found in another two microsatellite-stable tumors with ultrahigh TMBs. Two of these four tumors were from young patients (<50 years old, nonsyndromic), and there was seen a prominent T-cell infiltration in three of them. Furthermore, a somatic *POLE* p.A465T was found in a Lynch-associated tumor, which, hypothetically, might have enhanced TMB (which was the highest of all). In two tumors, a somatic *POLE* p.V411L and a *POLD1* p.E279K, respectively, were found only focally, and TMBs were low. It is commonly assumed that compromise of one allele is sufficient, but this has not been specifically addressed. Therefore, resequencing of the *POLE* or *POLD1* mutations was done with DNA from tumor cells isolated by laser-capture microdissection. This demonstrated that the mutations were monoallelic, and there was no evidence of a "second hit", neither by allelic loss (allelotyping with polymorphic microsatellite markers), nor by promoter methylation (Pyromark CpG assays). Taken together, including at least the more common DNA polymerase mutations in NGS panels allows for straightforward identification of hypermutator-type colorectal carcinomas which often may be "immunoreactive". This is important at least in young patients or when a metastasizing stage of disease has been reached and immune-checkpoint therapy enters deliberation.

Introduction

Mutations of the DNA polymerase ε gene (*POLE*) are an uncommon event in colorectal carcinomas, but their effects are, in many instances, profound and remarkable. Indeed, whole exome sequencing (WES) of 224 colorectal carcinomas by the TCGA project identified seven microsatellite-stable tumors that harbored *POLE* single nucleotide variants (3.1%) and carried a very high load of somatic gene mutations, in some cases even exceeding that of microsatellite-unstable tumors [1]. Based on these findings, the concept of the *POLE* mutated hypermutator-type of colorectal carcinoma as a separate molecular class was proposed, soon to be reinforced by similar observations reported for endometrial carcinoma [2].

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In humans, in addition to the DNA *POLE* there is known the DNA polymerase δ which is encoded by the *POLD1* gene. Both DNA polymerases are organized into two functionally distinct and evolutionary conserved domains, one that serves to incorporate nucleotides into the growing DNA strand (catalytic domain) and another that excises and substitutes correct nucleotides where infidelity in base-pairing has occurred (exonuclease domain). *POLE* single nucleotide variants associated with the hypermutator-type most often reside in the exonuclease domain (reviewed in [3]). However, a similar pattern so far has not been observed in sporadic colorectal carcinomas for *POLD1*.

Recognition of *POLE* mutated hypermutator-type colorectal and endometrial cancer has generated great interest among clinicians and researchers alike for two reasons. First, the high load of somatic mutations is expected to result in a wealth of neoantigens which, similiarly to microsatellite-unstable tumors, could make these tumors "immunoreactive" and, thus, amenable to immune-checkpoint inhibitor therapy, one of today's hot topics in oncology. Second, while in the TCGA series the *POLE* mutations appeared to be somatic events, only a year later a study by Palles et al. [4] of patients with early-onset colorectal carcinoma or intestinal polyposis demonstrated *POLE* or *POLD1* germline mutations in some patients. This, of course, has considerable implications for screening strategies of younger patients afflicted with either of these conditions, for genetic counseling, and for clinical approaches to molecular diagnostics.

Although the hypermutator class of colorectal carcinoma is well established by now some issues remain with the concept because biochemical analyses of exonuclease activity of *POLE* and *POLD1* single nucleotide variants as well as studies in model cell systems indicate a far less than straightforward mutation-function relationship [3]. This may mean that, in clinical specimens, the effects different *POLE* or *POLD1* mutations have on tumors may vary, and it provides a valid incentive to continue systematic DNA polymerase gene sequencing and detailed study of mutated cases. Information on these tumors is still limited and each new case is of value if underpinned by a complete array of clinical, morphological, and molecular data, tumor mutational burden (TMB), in particular. Furthermore, while it is generally assumed that *POLE* or *POLD1* needs to be compromised in one allele only to produce the hypermutator state, this has not been formally shown with clinical specimens because all published studies are based on tumor material that contained stroma alongside with tumor cells, which is a drawback to allelotyping analyses; and gene promoter methylation as a potential "second hit" has not at all systematically been assayed for.

This study was conceived to address the above issues. Specifically, we screened an unselected series of 271 colorectal carcinomas and investigated if the *POLE* and *POLD1* mutations found by this approach (i) belonged to clinicopathologically distinct tumors, those with an "immunoreactive" phenotype, in particular; (ii) conferred the hypermutator status on their tumor of origin; and (iii) were accompanied by a "hit" on the second allele of the mutated gene.

Materials and methods

Case selection and morphological studies

Screening for *POLE* and *POLD1* mutations was done on a series of 271 colorectal carcinomas selected from the Rostock branch of the North German Tumor Bank of Colorectal Cancer [5]. Patients' written informed consent was obtained as part of the tumor banking procedures, and studies on the archived tumor material were approved by internal board review (Ethics Committee of Rostock University, ref. II HV 43/2004). Routine pathological work-up of the resection specimens and basic molecular studies of the tumors (Bethesda panel microsatellite analyses and methylation analyses) were done as previously detailed [6]. This cohort for POLE and POLD1 mutation screening included 129 women and 142 men (mean age 68.07 years; median age 69 years; range 21–98 years; 14 patients younger than 45 years); for ten patients Lynch syndrome was diagnosed eventually.

The archived slides of the *POLE* and *POLD1* mutated tumors were reviewed to assess if there were morphological peculiarities that distinguished them from standard-type colorectal adenocarcinomas. Looking for the "immunoreactive" phenotype [7], CD8 immunohistochemistry (clone C8/144B, Dako, Glostrup, Denmark) was done, and densities of tumor infiltrating T cells were evaluated using QuPath [8] on digitized slides. For PD-L1 immunohistochemistry the E1L3N antibody (CST, Leiden, NL) was applied at 1:50 dilution after heat-induced antigen retrieval.

Clinical information on the patients was obtained by review of the charts of the Clinical Cancer Registry, University of Rostock.

POLE and POLD1 gene sequencing

DNA for *POLE* and *POLD1* sequencing was obtained by stereomicroscopically controlled microdissection of areas with viable tumor (>50%) from deparaffinised sections using a sterile scalpel blade. DNA was extracted as described in Hühns et al. [9]. Alternatively, snap-frozen samples that had been stored at −80 °C were used and DNA was extracted as published [6]. If snap-frozen tumor tissue was used, cryostat sections were prepared to ascertain that

the tumors were adequately represented by the sample (tumor content >50%). Normal DNA was from paraffin-blocks or snap-frozen samples of uninvolved colorectal mucosa at least 5 cm away from the tumors. DNA content was measured using the QuantiFluor® ONE dsDNA System (Promega, Madison, USA). Supplementary Table 1 lists which type of tissue (snap-frozen vs. paraffin-embedded) went into the different molecular studies.

Mutation screening of the exonuclease coding regions of *POLE* (exons 9–14) and *POLD1* (exons 7–10, 13) was performed by PCR amplification and Sanger sequencing. The primer sequences and PCR conditions are detailed in the Supplement (Supplementary Table 2). The sequence data were compared with reference sequences (*POLE*: ENSG00000177084, *POLD1*: ENSG00000062822) using SeqScape Software v2.7 (Applied Biosystems, Darmstadt, Germany).

Whole exome sequencing, somatic mutation analysis, and the assessment of tumor mutational burden

WES was done when screening by Sanger sequencing demonstrated a *POLE* or *POLD1* mutation. Except for tumor ID-T8, these studies were done with DNA extracted from tumor areas microdissected from paraffin sections as described above, and DNA was extracted using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For tumor ID-T8, snap-frozen tumor tissue was used, after preparing cryostat scout sections as described above DNA was extracted as published [6].

WES was performed as described previously [10]. Briefly, the SureSelect Human All Exon kit (Agilent, Santa Clara, USA) was used for target enrichment, and a HiSeq 4000 (Illumina, San Diego, USA) instrument for high-throughput DNA sequencing. Raw sequencing reads were converted to standard fastq format using bcl2fastq software 2.17.1.14, and fed to an in-house developed pipeline for the analysis of WES data that is based on the 1000 Genomes Project data analysis pipeline and GATK best practice recommendations. The short-reads were aligned to the GRCh37 (hg19) build of the human reference genome with bwa-mem with an average coverage of 245x and 95% of bases covered with at least 20 reads. Variant calling was performed using three different variant callers (GATK HaplotypeCaller, freebayes, and samtools).

Somatic variants were called from WES vcf files using the Sentieon-Genomics pipeline (sentieon-genomics-201808.03) which identified on average 12,000 high confidence somatic variants per sample. TMB was then calculated from high confidence somatic mutations which were located in exons and had a tumor allele frequency of 5% or greater. TMB levels were divided into three groups: low (<12 mutations/Mbp), high (>12 mutations/Mbp), and hypermutated (>100 mutations/Mbp).

POLE and POLD1 resequencing and allelotyping on DNA from laser-capture microdissected tumor samples

For laser-capture microdissection, 5 µm of cryostat or paraffin sections were mounted on polyethylene naphthalate (PEN) membrane-coated slides (MembraneSlide 1.0 PEN; Carl Zeiss Microscopy GmbH, Jena, Germany), fixed for 2 min in 70% ethanol and stained in hemalum for 15 s. Subsequently, the slides were rinsed in water for 2 min and dehydrated in 70%, 96 and 100% ethanol, each for a minute. After air-drying, slides were stored at −20 °C until use. Laser-capture microdissection was performed on a Zeiss Axio Observer (Carl Zeiss) using the RoboLPC method as implemented with the device to microdissect and capture the areas of interest. Tumor samples were collected into 0.5 ml adhesive caps and DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's instructions.

First, the DNA was used for resequencing the *POLE* or *POLD1* mutations seen by screening. Second, allelic imbalances (AI) were assayed for with polymorphic microsatellite markers that map to chromosomal regions close to *POLE* (D12S357, D12S1638, D12S1723) or *POLD1* (D19S246, D19S585, D19S601, D19S867, D19S904, D19S907) as described in Church et al. [11]. Tumor and nontumor DNA was amplified by PCR as follows: reaction were started at 95 °C for 1 min and this was followed by 30 cycles at 95 °C for 15 s, 52 °C (only for D12S1638, D12S1723) or 55 °C for 15 s, 72 °C for 10 s. PCR products were separated by capillary gel electrophoresis and detected on an automated ABI 3500 Genetic Analyzer (Applied Biosystems). Fragment length and fluorescence intensity were evaluated by Gen′eMapper software (Applied Biosystems). The GeneScan™-500LIZ™ size marker (Applied Biosystems) served as an internal standard. AI was scored positive if the tumor/normal ratio was <0.5 or >2.0.

Methylation analyses

These studies were done with DNA extracted from tumor that was microdissected from paraffin sections as described above. Bisulfite conversion was carried out using the Epitect Bisulfite Kit (Qiagen) according to the manufacturer's recommendations. CpG promoter methylation analyses of *POLE* and *POLD1* was performed with a Q24 Pyromark instrument (Qiagen). A novel Pyromark CpG assay was developed for the *POLE* promoter with the Pyromark Assay

Table 1 Complete list of clinical, morphological, and molecular data of colorectal carcinomas detected with *POLE* or *POLD1* mutations.

Case	ID-T6	ID-T2	ID-T5	ID-T1	ID-T4	ID-T3	ID-T9	ID-T7	ID-T8
Clinicopathological data									
Gender	Male	Male	Female	Male	Female	Female	Female	Female	Female
Age (years)	36	57	41	62	55	76	21	66	41
Tumor localization	Ascending colon	Cecum	Transverse colon	Ascending colon	Cecum	Rectum	Ascending colon	Ascending colon	Ascending colon
UICC stage	II	III	I	II	III	IV	II	IV	III
Overall survival	Alive	Alive	Alive	DOD at 65 years	DOD at 56 years	Alive	Alive	DOD at 68 years	DOD at 47 years
Morphology									
Histology	Tubular/tubulovillous	Heteromorphous: tubular to solid	Tubular/cribriform	Solid/medullary, nuclear anaplasia	Heteromorphous: cribriform/solid-medullary; mucinous component	Tubulovillous	Sarcomatoid	Tubular/ cribriform/ solid; nuclear anaplasia	Tubular/ cribriform
Grade	2	3	2	3	3	2	4	3	2
CD8-T (mm^2 tumor)	High: 179	Low: 35	High: 323	High: 438	High: 225	High: 464	n.d.	Low: 55	High: 535
CD8 centre (mm^2 total area)	Intermediate: 155	Low: 48	High: 403	High: 423	High: 238	High: 592	Low: 37	Low: 58	High: 637
PD-L1	Negative	Negative	Negative	Negative	Positive (<1%)	Negative	Negative	Negative	Negative
Molecular data									
Molecular class	spSTD	spSTD	spSTD	spSTD	LS (MSH6 p. K1140Q)	spSTD	spSTD	spSTD	LS (MLH1 p. L296ter)
POLE variant—Sanger screen	p.P286R(s)	p.P286R(s)	p.V411L(s)	p.V411L(s)	p.A465T(s)	p.D287E(g)	–	–	–
POLE variant—WES	p.P286R(s),	p.P286R(s), p. F959L(s),	p.V411L(s)	p.V411L(s),	p.A465T(s); p. A252V(g),	p.D287E(g)	Het: p.V411L(s)	p.A252V(g),	p.N1396S(g)
	p.R1556W(s)	p.P2088S(g)		p.S1380L(s)	p.S38C(g), p. N1396S(g)			p.N1396S(g)	
POLD1 variant—Sanger screen	–	–	–	–	–	–	Het: p.R525Q(s)	Het: p.E279K(s)	p.L342M(s)
POLD1 variant—WES	–	–	–	–	–	–	–	–	p.L342M(s)
TMB (variants/ Mbp)	Ultrahigh: 275.33	Ultrahigh: 302.73	Ultrahigh: 125.9	Ultrahigh: 199.17	Ultrahigh: 310.83	Low: 3.7	Low: 5.03	Low: 6.6	High: 32.4
Methylation studies							ND		
POLE promoter (PMR)	T: 2.8/N: 0.6	T: 3.0/N: 1.5	T: 2.5/N: 2.3	T: 3.3/N: 2.5	T: 4.5/N: 3.6	T: 2.8/N: 1.8	ND	n.a.	n.a.

Table 1 (continued)

Case	ID-T6	ID-T2	ID-T5	ID-T1	ID-T4	ID-T3	ID-T9	ID-T7	ID-T8
POLD1 promoter (PMR)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	T: 4.8/N: 3.5	T: 5.5/N: 5.8
Laser-capture studies									
POLE/POLD1 mutation status	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Monoallelic	Monoallelic	ND	Monoallelic	Monoallelic
D12S357 (POLE allelotyping)	no AI	no AI	no AI	no AI	no AI	no AI	ND	n.a.	n.a.
D12S1638 (POLE allelotyping)	no AI	no AI	no AI	no AI	no AI	no AI	ND	n.a.	n.a.
D12S1723 (POLE allelotyping)	no AI	no AI	no AI	no AI	no AI	no AI	ND	n.a.	n.a.
D19S246 (POLD1 allelotyping)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	AI	no AI
D19S585 (POLD1 allelotyping)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	no AI	MSI
D19S601 (POLD1 allelotyping)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	no AI	no AI
D19S867 (POLD1 allelotyping)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	no AI	MSI
D19S904 (POLD1 allelotyping)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	no AI	no AI
D19S907 (POLD1 allelotyping)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	ND	no AI	MSI

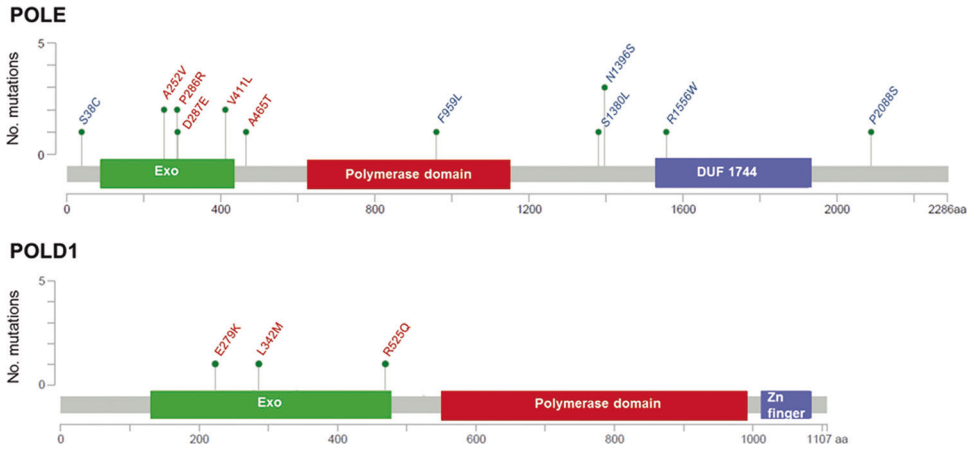

Fig. 1 "Lollipop" graphic of *POLE* and *POLD1* missense variants. Variants found by Sanger sequencing are in red letters, variants found in addition by WES are in blue.

Design 2.0 software (Qiagen). The assay addressed methylation in six CpG sites within the *POLE* promoter (chr12: 132687510-132687530) by PCR as follow: forward primer: 5′-GGTAGGGATAGGGGAAAGTG-3′, reverse primer: 5′ (5-biotin labeled)-ACCCACCCCACCTCAAAC TAACC-3′, sequencing primer: 5′GGAAAAGTGTGTGG TAG-3′. The Pyromark Hs_POLD1_01_PM PyroMark CpG assay (Qiagen) was used for methylation analyses of the *POLD1* promoter according to the manufacturer's recommendations. Target CpGs were evaluated by Pyromark Q24 2.0.7 software (Qiagen), which converts the pyrograms to numerical values for peak heights and calculates the proportion of methylation. Methylation values <5% were considered as background. Hypermethylated DNA provided by the manufacturer was used as standard control.

Results

POLE and POLD1 mutations detected in this series

An unselected series of 271 primary colorectal carcinomas was screened by Sanger sequencing for *POLE* mutations in exons 9–14 and *POLD1* mutations in exons 7–10 and exon 13, as detailed in "Materials and methods" section.

By this screening approach, we identified six tumors with nonsynonymous *POLE* variants (Table 1). Specifically, we found the single nucleotide variants p.P286R (tumors ID-T6 and ID-T2), p.D287E (tumor ID-T3), p.V411L (tumors ID-T5 and ID-T1), and p.A465T (tumor ID-T4), which are all on record in the COSMIC database or have been reported previously in the literature [12]. All mutations were located in the exonuclease domain of the polymerase (Fig. 1), and for all of them a significant effect on protein function was predicted by in silico algorithms (PolyPhen, MutationTaster, PROVEAN, SIFT; Table 2). The variants p.P286R, p.V411L, and p.A465T in the tumors were somatic observations, as assaying DNA extracted from normal mucosa demonstrated wildtype genotypes. However, the p.D287E variant was also found in the mucosa specimen and in nontumor liver tissue from a resection performed later on, and thus apparently was present in the patient's germline. All these *POLE* variants were recovered by WES, which in four of the six tumor samples also yielded a total of seven additional single nucleotide variations outside the exonuclease domain (see Fig. 1 and Table 1).

By Sanger sequencing of *POLD1*, three tumors with variants were found as follows (Fig. 1 and Table 1): a single nucleotide variant p.E279K (in tumor ID-T7), a variant p.L342M (in tumor ID-T8), and a variant p.R525Q (in tumor ID-T9; details in Table 2). All these variants were somatic and were located in the exonuclease domain (Fig. 1). However, in silico predictions as to their functional effects were mixed (Table 2): the p.E279K variant was classified as nonsignificant by all four algorithms, whereas a compromise of gene function was predicted for the variants p.L324M and p.R525Q by one and two of the four algorithms, respectively. WES of the cases with *POLD1* variants confirmed the p.L342M in tumor ID-T8. However, WES of tumors ID-T7 and ID-T9 led to two unexpected observations: First, in tumor ID-T7, the variant p.E279K mutation was not recovered. To clarify this discrepancy, Sanger resequencing was done with the DNA used for the initial screen as well as with the DNA used for WES. Resequencing demonstrated the p.E279K in the initial screening sample, but not in the sample used for WES, which indicates a heterogeneous distribution of this variant within this tumor. Second, in tumor ID-T9, the *POLD1* p.R525Q variant was not recovered by WES, which, however, demonstrated a *POLE* p.V411L variant that had not been picked up by Sanger sequencing. To exclude technical issues, Sanger resequencing was done with the DNA sample used for WES, and this clearly demonstrated the *POLE* p.V411L whereas the *POLD1* p.R525Q was absent from this sample. Thus, *POLE* and *POLD1* mutations in tumor ID-T9 appeared to be heterogeneous.

Table 2 Results from in silico algorithm testing of the nonsynonomous POLE and POLD1 missense variants detected in this study.

Tumor(s)	Gene	Amino acid change	Nucleotide change	Domain	Somatic vs. Germline	Detected by	Additional mutation(s) found in the tumor	PolyPhen	MutationTaster	PROVEAN	SIFT	COSMIC
ID-T4	POLE	S38C	c.112A>T	Outside	Germline	WES	p.A252V, p.A465T, p.N1396S	0.01 (Benign)	Disease causing	Neutral	Damaging	–
ID-T4, ID-T7	POLE	A252V	c.755C>T	Exonuclease	Germline	WES	ID-T4: p.S38C, p.A465T, p.N1396S; ID-T7: p.N1396S	0.007 (Benign)	Polymorphism	Neutral	Tolerated	COSM5880915
ID-T2, ID-T6	POLE	P286R	c.857C>G	Exonuclease	Somatic	Sanger, WES	ID-T2: p.F959L, p.P2088S; ID-T6: p.R1556W	1.0 (Probably damaging)	Disease causing	Deleterious	Damaging	COSM937333
ID-T3	POLE	D287E	c.861T>A	Exonuclease	Germline	Sanger, WES		1.0 (Probably damaging)	Disease causing	Deleterious	Damaging	–a
ID-T1, ID-T5; ID-T9 (het)	POLE	V411L	c.1231G>C	Exonuclease	Somatic	Sanger, WES	ID-T1: p.S1380L; ID-T9: POLD1(het) p.R525Q	1.0 (Probably damaging)	Disease causing	Deleterious	Damaging	COSM4716440
ID-T4	POLE	A465T	c.1393G>A	Exonuclease	Somatic	Sanger, WES	p.S38C, p.A252V, p.N1396S	1.0 (Probably damaging)	Disease causing	Deleterious	Damaging	COSM6834810
ID-T2	POLE	F959L	c.2877C>A	Polymerase	Somatic	WES	p.P286R, p.P2088S	1.0 (Probably damaging)	Disease causing	Deleterious	Damaging	–
ID-T1	POLE	S1380L	c.4139C>T	Outside	Somatic	WES	p.V411L	0.001 (Benign)	Polymorphism	Neutral	Tolerated	COSM937287
ID-T4	POLE	N1396S	c.4187A>G	Outside	Germline	WES	p.S38C, p.A252V, p.A465T	0.068 (Benign)	Polymorphism	Deleterious	Tolerated	COSM3753066
ID-T6	POLE	R1556W	c.4666C>T	DUF1744	Somatic	WES	p.P286R	0.998 (Probably damaging)	Disease causing	Deleterious	Damaging	COSM1360259
ID-T2	POLE	P2088S	c.6262C>T	Outside	Germline	WES	p.P286R, p.F959L	0.902 (Possibly damaging)	Disease causing	Deleterious	Damaging	COSM3871127
ID-T7 (het)	POLD1	E279K	c.835G>A	Exonuclease	Somatic	Sanger	POLE p.A252V, p.N1396S	0.0 (Benign)	Polymorphism	Neutral	Tolerated	–
ID-T8	POLD1	L342M	c.1024C>A	Exonuclease	Somatic	Sanger, WES	POLE: p.N1396S	0.014 (Benign)	Disease causing	Neutral	Tolerated	–
ID-T9 (het)	POLD1	R525Q	c.1574G>A	Exonuclease	Somatic	Sanger	POLE: p.V411L (het)	0.274 (Benign)	Disease causing	Neutral	Damaging	–

[a]Described in Jansen et al. [12]

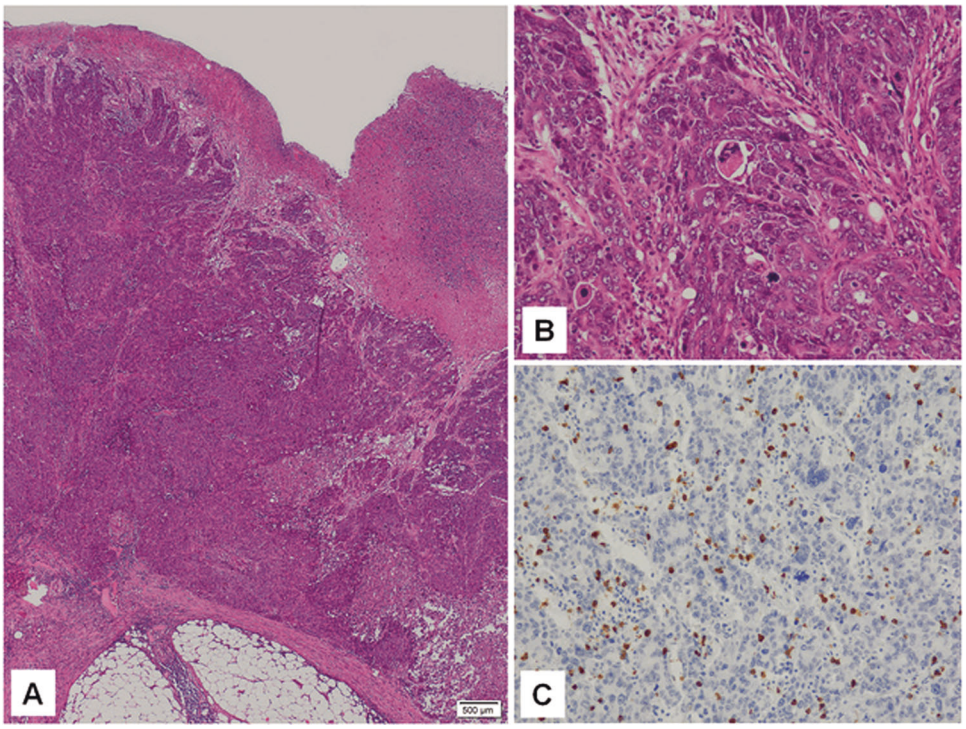

Fig. 2 Microphotographs of tumor ID-T1. This poorly differentiated colorectal carcinoma had a somatic *POLE* variant p.V411L. Note the "pushing" tumor border (arrows in **a**) and the anaplastic tumor cell nuclei (asterisks in **b**). Infiltration by $CD8^+$ T cells was prominent (**c**).

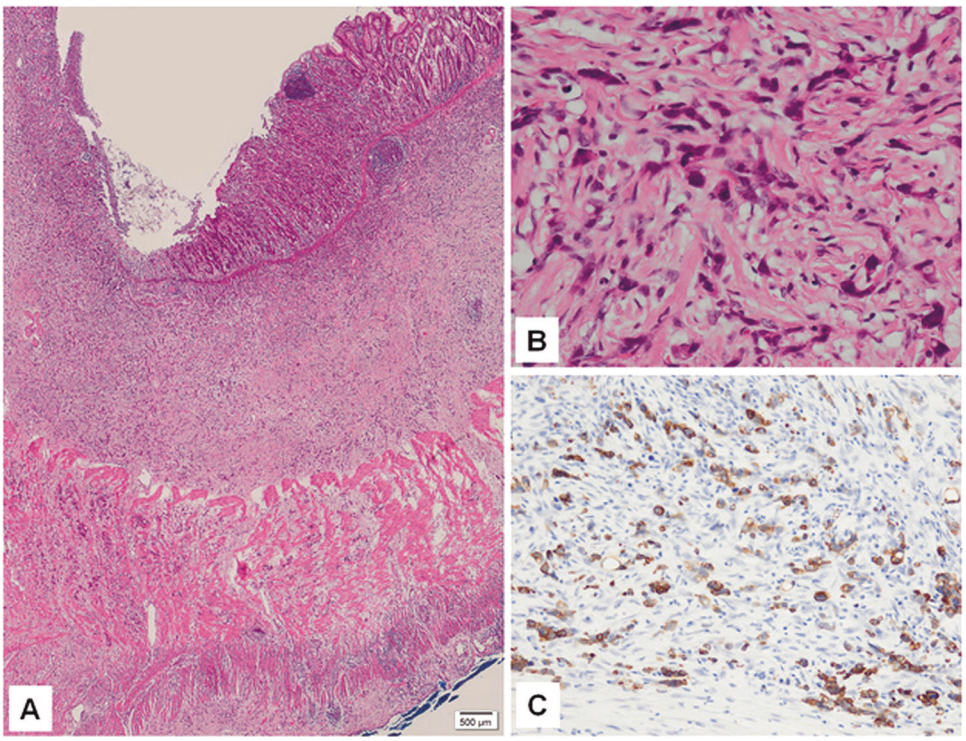

Fig. 3 Microphotographs of tumor ID-T9. This highly unusual colorectal carcinoma from a 21-year-old nonsyndromic patient harbored simultaneous, albeit hetereogeneous, a somatic *POLE* variant p.V411L and a somatic *POLD1* p.R525Q. Note the almost diffuse mode of infiltration (**a**) and the sarcomatoid features of the cancer cells (**b**). CK20 was positive by immunohistochemistry (**c**), as was CDX2 (not shown).

Clinical features of patients and morphological features of POLE and POLD1 mutated colorectal carcinomas

Information on clinical characteristics of the patients with *POLE* or *POLD1* mutated tumors as well as morphologic and molecular features are detailed in Table 1. Notably, two patients' tumors were microsatellite-unstable, one with a somatic *POLE* p.A465T variant and one with a somatic *POLD1* p.L342M variant (tumors ID-T4 and ID-T8 in Table 1). Eventually, Lynch syndrome could be demonstrated in these patients because the tumors were seen to

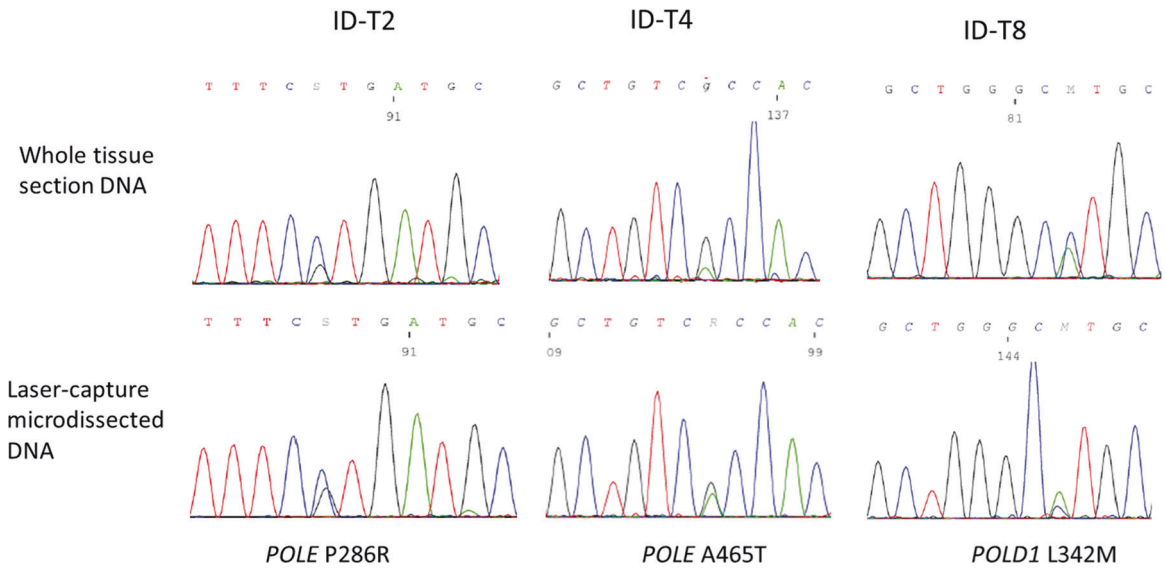

Fig. 4 Examples of Sanger sequencing on DNA obtained by laser-capture microdissections from three tumors with *POLE* or *POLD1* mutations.

lack expression of the mismatch repair proteins MSH6 or MLH1 by immunohistochemistry, and germline mutations of these genes were found in the course of further genetic work-up of the patients (MSH6: p.K1140Q and MLH1: pL296ter, respectively). Histologically, both tumors were heterogenous in their composition, areas with mucinous patterns alternating with solid to medullary areas and numerous stromal and intraepithelial T cells, which, overall, is the classical morphology of Lynch syndrome-associated colorectal carcinomas. A minority of tumor cells (<1%) was PD-L1 positive in ID-T4 whereas ID-T8 remained negative.

The remaining tumors were microsatellite-stable. Of these, two *POLE* mutated tumors were histologically unusual: ID-T1 and ID-T2 were composed of large solid nests or strands of tumors cells with considerable nuclear anaplasia; the tumor borders were "pushing" and tumor budding and the stromal desmoplasia commonly observed in colorectal carcinomas were inconspicuous. In ID-T1, nuclear anaplasia was quite striking (Fig. 2). By CD8 immunohistochemistry, a dense T-cell infiltrate was seen in ID-T1. Tumor cells were PD-L1 negative by immunohistochemistry, although immunostaining of peritumoral histiocytes was seen in all cases.

ID-T9, the tumor with the heterozygous *POLE* p.V411L and *POLD*1 p.R525Q variants, was highly unusual: histologically, it was a sarcomatoid cancer (Fig. 3), which is an exceptional phenotype for a primary colorectal carcinoma, and, equally unusual, the patient was only 21 years old. Tumor infiltrating T cells were sparse in this cancer, and PD-L1 was not expressed. The rest of the tumors (ID-T3, ID-T5, ID-T6 with *POLE*, and ID-T7 with *POLD1* variants) were moderately differentiated, non-mucinous, standard-type colorectal adenocarcinomas. T cell infiltration was prominent in all these tumors, but PD-L1 was not expressed by the tumor cells.

Association of POLE and POLD1 gene mutations with tumor mutational burden

Remarkably high mutational rates could be demonstrated by WES in five of the seven tumors with *POLE* mutations (Table 1). In these, mutational rates ranged from 126 mutations/Mbp of genomic DNA to 311 mutations/Mbp, which puts these tumors well into the class of "hypermutators" (ID-T1, ID-T2, ID-T4, ID-T5, and ID-T6 of Table 1). However, mutational rates were low in ID-T3, the germline *POLE* variant p.D287E carrier's tumor, and in ID-T9, the tumor with the heterozygous *POLE* p.V411L and POLD1 variant p.R525Q. The TMB was also low in tumor ID-T7 which harbored the heterogeneous *POLD1* variant p.E279K. A high mutational rate of 32.4 mutations/Mbp was recorded for the third *POLD1* mutated tumor (ID-T8), one of the two Lynch syndrome-associated cancers.

Resequencing and allelotyping on tumor DNA obtained by laser-capture microdissection and promoter methylation studies

To exclude the confounding effect of nontumor DNA from the analyses, resequencing and allelotyping was made with DNA that was extracted from tumor cells isolated by laser-capture microdissection. These analyses could be completed successfully for tumors ID-T1 through to ID-T8, but ID-T9, the sarcomatoid carcinoma, due to its growth pattern was not amenable to laser-capture microdissection. First, we used the DNA to resequence the *POLE* or *POLD1* variants,

respectively. We observed that all variants were heteroallelic (Fig. 4). Second, we addressed if the *POLE* or *POLD1* gene variants were combined with loss of the second allele. PCR-based allelotyping studies with polymorphic microsatellite markers were informative with at least some markers in all our cases (Table 1). Allelic loss, which on laser-capture microdissected material would have resulted in complete loss of one allele in the electropherograms, was not seen in any of the cases. There was seen a moderate allelic imbalance with one of six markers for the *POLD1* mutated tumor ID-T7, but not for the remaining five markers (Table 1). In tumor ID-T8, as can be expected for a Lynch syndrome-associated cancer, microsatellite instability interfered with allelotyping in three of the six markers, but the remaining assays were informative and without indication of allelic loss.

Finally, *POLE* or *POLD1* promoter methylation studies were done, which did not demonstrate methylation in any of our cases (Table 1).

Discussion

Colorectal carcinomas with *POLE* or *POLD1* mutations have been recognized not very long ago as a separate molecular class in which the extremely high numbers of gene mutations (the hypermutator molecular phenotype) are a salient and defining feature [1, 13]. Colorectal carcinomas were first in this concept, endometrial carcinoma to be included not much later, and by now similar observations have been reported for other types of cancer too [14]. However, hypermutator-type colorectal carcinomas are rare. Finding them requires technology quite beyond what currently is used for standard pathological work-up of surgical pathology specimens. Therefore, published information on them is still limited. It remains a topic of interest in surgical pathology how *POLE* or *POLD1* mutated colorectal carcinomas are set off from the rest by clinical, morphological, and/or molecular features.

In this study, we screened colorectal carcinomas obtained by surgery from a consecutive series of unselected patients for *POLE* or *POLD1* mutations in the exonuclease domains. Screening 271 tumors yielded six *POLE* mutated and three *POLD1* mutated tumors (2.2% and 1.1% of the series, respectively), an overall frequency similar to that observed in previous series [1, 14].

A somatic *POLE* variant p.P286R was found in two of our cases (ID-T6 and ID-T2). Both were microsatellite-stable and carried a very high load of mutations (TMB of 275 and 303, respectively). The *POLE* variant p.P286R is one of the by now well-appreciated driver mutations of the *POLE* gene. Indeed, features of *POLE* p.P286R mutated colorectal carcinomas of the hypermutator-type have recently been reported by Ahn et al. [15] who identified them by WES in four of 28 patients diagnosed at an age <40 years in a first exploratory series, adding another six cases by Sanger sequencing of *POLE* codon 286 of 83 microsatellite-stable colorectal carcinomas from patients aged <50 years at the time of diagnosis. Based on these findings, these authors propose that they represent a separate class of early-onset colorectal carcinomas. Histomorphological features, however, were reported as largely uncharacteristic (except for, as a casual observation, frequent debris of necrotic/apoptotic cells mingled with granulocytes in the lumens where tumors were cribriform), and T-cell infiltration was low. Contrary to the Ahn et al. observations, our ID-T2 was from a patient aged 57 at the time of diagnosis which is not really early-onset, anymore; and ID-T6 was observed to be "immunoreactive", at least by our criteria [7].

The *POLE* variant p.V411L, yet another established *POLE* driver-mutation, was found in our Sanger sequencing screen in two microsatellite-stable tumors (ID-T1 and ID-T5). As would be expected, TMBs were very high in both cases (199 and 126 variants/Mbp, respectively). Furthermore, quite in line with the idea that hypermutation due to a wealth of neoantigens can elicit an enhanced antitumor immune response, in both tumors T-cell infiltration was very high: intraepithelial T cells were recorded well above the threshold of 123 $CD8^+$ T cells per square millimeter of tumor (our criterion for "immunoreactive" tumors; 7); T cell infiltration per square millimeter total in the tumor centre was around 400, which according to Domingo et al. amounts to a high density [16]. To be pointed out as additional unusual features, ID-T5 was from an unusually young patient (aged 41 years), and ID-T1 was notable for a striking nuclear anaplasia, reminiscent of what has been reported for *POLE* mutated endometrial carcinomas [17].

ID-T9 of our series puts our current understanding of the role of *POLE* mutations to a test. The patient was only 21 years old at the time of diagnosis which would be unusual even for a syndromic colorectal cancer. However, genetic work-up did not provide any evidence for an association with a known cancer predisposition syndrome. Furthermore, the tumor was sarcomatoid by histology, which is a rarity among colorectal carcinomas. Secondary involvement of the colon was excluded by the tumor's macroscopic features (growth from the lumen towards the mesocolic fat), staging examinations (no other tumor found), and immunohistochemistry (CDX2 and CK20 positive, CK7 negative). As regards the DNA polymerase gene mutations, the tumor was unusual for simultaneous *POLE* and *POLD1* mutations (p.V411L and p.R525Q, respectively), both of which, however, were not present throughout the tumor. Heterogeneity of these mutations within the tumor may explain why TMB was low because the *POLE* variant p.V411L, at least if present throughout, most likely would

have resulted in a hypermutator-type cancer. As far as we are aware, heterogeneity of DNA polymerase gene mutations has not been addressed by any published studies. In our study, mutational heterogeneity was a coincidental finding that came to light when trying to reconcile the Sanger screen data with the WES data, but it may be more common than anticipated and add an unexpected layer of complexity. Indeed, the *POLD1* variant p.E279K found in ID-T7 was a second example of mutational heterogeneity in our series, although this mutation may well be a passenger-type mutation that, even if uniformly present, may not lead to a hypermutator state.

Assessing the biological significance of DNA polymerase gene mutations is quite difficult in many instances. In vivo studies suggest that mutator effects may depend on the specific mutation incurred by a tumor, which may be weak/moderate to strong in terms of TMB (reviewed in 3). Well-appreciated, recurrent mutations with strong effects on TMB are few, the *POLE* variants p.P286R and p.V411L found in five of our mutated tumors among them. However, the remaining *POLE* and *POLD1* mutations of our series are not straightforward to place, this being compounded in some cases by the fact that more than one *POLE* or *POLD1* variants could be demonstrated once WES was completed (Table 1). Basically, there are three possible approaches to assessing the functional role of the mutations. First, in silicio algorithms that predict the mutations' effects on gene function can be employed (as shown in Table 2). By these algorithms, as to be expected, *POLE* variants p.P286R and p.V411L were clearly flagged as functionally relevant. However, the *POLE* variant p.D287E was read out as functional by all four algorithms, but the TMB was low in the corresponding tumor and the variant was germline in a 76-year-old woman without polyposis, which makes this in silicio assessment implausible. *POLE* p.F959L, p.R1556W, and p.P2088S were three more missense variants that were predicted to be functionally compromising by all algorithms. Nevertheless, it remains difficult to draw a final conclusion regarding their functional roles because they all targeted *POLE* in regions outside the exonuclease or polymerase domains (Fig. 1) and they were found in the two tumors with a p.P286R mutation which by itself would explain the high TMBs. The *POLD1* p.L342M of tumor ID-T7, scored as functionally relevant by the MutationTaster algorithm (although not by the other three), was associated with a high, but not ultrahigh TMB. However, this most likely can be ascribed to the mismatch repair deficiency recorded for this tumor. Finally, *POLE* p.A465T found in tumor ID-T4, the last of the functionally important missense variants by in silicio testing, remains difficult and interesting. For its assessment, as a second approach to the issue of function, we conferred our list with the list of *POLE* and *POLD1* mutations published by Campbell et al. [14] whose "in vivo human mutagenesis screen" of a formidable 81,337 cancers of various types affords an impressive resource. Indeed, the *POLE* variant p.A465T in tumor ID-T4 is reported by them as associated with an ultrahigh mutational burden in two tumors (their Supplementary Table 2), although it is not included in their list of 29 *POLE* driver mutations (their Supplementary Table 3). The interpretation of the *POLE* variant p.A465T's significance in our case is complicated by the fact that this tumor was Lynch syndrome-associated, which by itself might explain hypermutation. Conceivably, the *POLE* variant p.A465T might put a mutational load on top of that exacted by the mismatch repair deficiency, conceptualized as an "explosive mutation accumulation" by Hodel et al. [18]; consistent with this idea TMB of this tumor was highest of all. It may be added, parenthetically, that finding a *POLE* mutation in combination with mismatch repair deficiency is exceptional because usually microsatellite instability in colorectal carcinomas combines with *POLD1*, but not *POLE* mutations. The remaining mutations to be clarified are not found in the Campbell et al. files. Finally, a third and, obviously, most valid approach to assessing the significance for *POLE* or *POLD1* mutations on TMB derives from model cell systems. However, the list of *POLE* and *POLD1* mutations studied in this way remains short, so far, and our mutations in question are not among them [3].

A second part of our study addressed the state of the second allele in our *POLE* or *POLD1* mutated colorectal carcinomas. This is a topic of interest because it is well appreciated that *POLE* and *POLD1* are not tumor suppressor genes in a classical sense: on one hand, all *POLE* and *POLD1* mutations associated with hypermutator-type tumors (driver mutations) on record are point mutations whereas truncating mutations or frameshifts are quite rare and, if present, appear to be passenger mutations; on the other hand, the point mutations of *POLE* and *POLD1* in hypermutator-type tumors confer loss-of-function, and therefore, obviously, the genes cannot be classified as oncogenes, either. Conceivably, mutated *POLE* and *POLD1* are "hybrids" and defy to be placed neatly in the oncogene-tumor suppressor gene dichotomy. In the experimental setting, a heterozygous state of *POLE* or *POLD1* mutations is sufficient to induce and/or maintain high mutational burdens in cell lines derived from hypermutator tumors or yeast systems [19–21]. However, the situation is less clear in clinical tumor specimens which to-date have only been studied with DNA derived from tumor homogenates. Therefore, we performed laser-capture microdissection to separate tumor cells from the surrounding stroma and used the DNA thus obtained for resequencing and for allelotyping with polymorphic microsatellite markers. In addition, we tested for gene promoter methylations, another mechanism by which the second allele could be

compromised in its function. We observed that all *POLE* and *POLD1* mutations were monoallelic, and there was no evidence of a "second hit" on the genes, neither by allelic loss, nor by promoter methylation. These findings suggest that simply the 50% reduction of *POLE* gene dosage caused by the variants p.P286R or p.V411L, respectively, are sufficient in conferring ultrahigh TMBs in sporadic colorectal carcinomas.

Taken together, in this study we have added evidence to the rising concept that *POLE* variants p.P286R and p.V411L in colorectal carcinomas define a separate class, although heterogeneity may occasionally prove an issue to be kept in mind. These tumors are not identified in a straightforward fashion by histology, but very likely are "immunoreactive". Thus, at least these *POLE* hotspots (and the few others not found here) should be included into sequencing panels, especially if dealing with young patients and/or patients with metastasizing disease in case immune-checkpoint therapy is deliberated. Of the less frequent mutations, *POLE* p.D287E was not associated with high TMB, but *POLE* p.A465T may be relevant, although in our series this may have been obscured by the additional mismatch repair defect. As an aspect of tumor biology addressed here, we have shown that a "second hit" on *POLE* is not required for a significant functional effect.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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