Clinical and Experimental Medicine (2019) 19:393–400
https://doi.org/10.1007/s10238-019-00558-7

ORIGINAL ARTICLE

Mutation analysis of *POLE* gene in patients with early-onset colorectal cancer revealed a rare silent variant within the endonuclease domain with potential effect on splicing

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Received: 25 March 2019 / Accepted: 23 April 2019 / Published online: 2 May 2019

Abstract

The colorectal cancer harbor germline, somatic or epimutations in mismatch repair genes, *MUTYH* or *POLE* gene, which lead to the hypermutated and ultramutator phenotypes with increased immune response. The mutations in *POLE* gene were reported to occur more frequently in early-onset colorectal cancer (EOCRC), and the patients are strong candidates for checkpoint inhibitor therapy. Here, we report mutation analysis within the endonuclease domain of the *POLE* gene in the cohort of patients with EOCRC in order to identify recurrent or new mutations and evaluate their association with the presence of tumor-infiltrating lymphocytes (TILs) and peritumoral lymphoid reaction. We have shown a significant association between MSI tumors and TILs ($p = 0.004$). Using sensitive single-tube nested PCR with subsequent Sanger sequencing, we have found in one female patient diagnosed at age 48 with rectal adenocarcinoma with mucinous elements staged pT3pN2pM1 a silent variant within the exon 9 NM_006231.3 c.849 C > T, NP_00622.2 p.Leu283 = recorded in dSNP as rs1232888774 with MAF = 0.00002. In silico prediction, result showed possible involvement into splicing; therefore, this rare variant can be involved into EOCRC pathogenesis. In the time of precise medicine, it is important to develop screening strategies also for less common conditions such as EOCRC allowing to predict tailored therapy for younger patients suffering from CRC that harbor mutations in the *POLE* gene.

Keywords Early-onset colorectal cancer · *POLE* mutations · Microsatellite instability · Tumor-infiltrating lymphocytes

Introduction

Colorectal cancer (CRC) belongs to the most common human cancers and represents the second main cause of death from cancer worldwide [1].The results published by

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The Cancer Genome Atlas (TCGA) network showed that about 16% of CRC are positive for microsatellite instability (MSI) whose consequence is the accumulation of mutations in the DNA of tumor cells resulting in hypermutated tumors [2]. Patients with MSI harbor germline, somatic mutations or epimutations in genes coding for mismatch repair (MMR) [3]. Among patients that are microsatellite stable (MSS), increased number of somatic mutations was reported in patients with MUTYH-associated polyposis with deficient base extinction repair [4, 5]. The other genetic mechanism leading to ultramutator phenotype in MSS CRC with higher mutation load than those of hypermutated cancers is caused by germline mutations in *POLE* or *POLD1* conferring high penetrance predisposition for CRC referred to as proofreading-associated polyposis, and also by somatic mutations in *POLE* gene [2, 6, 7]. Similarly, the somatic mutations in *POLE* gene have been found in endometrial cancer (EC) with ultramutator phenotype and represent also an uncommon phenotype in non-small cell lung cancer (NSCLC) [8–10]. The mutations occur mainly within the exonuclease domain (ED) of the protein, which is associated with the proofreading function of the polymerase. The frequencies of *POLE* mutations in EC and CRC comprise 6–12% and about 0.65–1%, respectively, and are associated with very good prognosis [8, 9, 11–13]. *POLE*-mutated endometrial cancers exhibit high neoantigen load and tumor-infiltrating lymphocytes (TILs) with high expression of PD-1 and PD-L1 considering these mutations as predictive for the therapy with checkpoint inhibitors [14]. Therapy with checkpoint inhibitors showed significant responses in patients with CRC and in one patient with MSS and *POLE*-mutated CRC [15, 16]. The identified recurrent mutations within the ED account for 89.4% of proofreading domain mutations and are more frequent in younger patients. These mutations were present in 4.2% patients younger than 50 years [12] and in 7.2–7.6% cases with less than 40 years old at diagnosis [12, 17].

The CRC is prevalent in age groups over 50 years; however, the recent data are shown a rising incidence of invasive CRC among patients aged mostly less than 50, who are classified as early-onset CRC (EOCRC) [18]. About 80–85% of EOCRC patients do not harbor germline mutations in high penetrant genes and about 50% are without family history [19]. Therefore, the increased role of environment and lifestyle-related factors can be considered as weighty triggers for the early onset of the disease, and the identification of early driver events is substantial for the understanding of the pathogenesis of this group of patients [20]. Recently, it was shown that *POLE* mutations represent an early event in sporadic endometrial and colorectal cancer [21] and their role in EOCRC remains to be elucidated. The aim of our study was to implement sensitive nested PCR with successive Sanger sequencing in order to analyze clinical samples from patients with EOCRC for mutations within ED of the *POLE* gene and to test the hypothesis that the recurrent *POLE* mutations are more frequent in patients with EOCRC. The second aim of the study was the identification of patients with mutations in ED of the *POLE* gene that could benefit from the checkpoint inhibitor therapy and study the tumor lymphocyte infiltration and peritumoral lymphoid reaction.

Materials and methods

Patients and the identification of TILs

In this study, 39 patients with the diagnosis of CRC, who were participants from the previous prospective study of 270 CRC patient cohort, were enrolled [22]. The inclusion criteria were age 52 years or less at diagnosis and histologically confirmed diagnosis of CRC. The study was approved by ethical committee, and informed consent was obtained from the participants. The standard clinicopathological parameters are shown in Table 1. The mean age of patients comprises 44.2 years at the diagnosis with median age of 46. Paraffin-embedded tissue (FFPE) section was obtained from biopsy specimens of CRC. The histological assessment was performed on HE-stained FFPE sections,

Table 1 Clinicopathological characteristics of the patients

Patients	39
Age	
Range	30–52
Mean	44.2
Median	46
Gender	
Male	21
Female	18
Histological type	
Adenocarcinoma	32
Mucinous adenocarcinoma	5
Adenocarcinoma with	
Mucinous elements	1
Non differentiated	1
Tumor localization	
Right (cecum, ascending colon, hepatic flexure and transverse colon)	13
Left (splenic flexure, descending colon, sigmoid colon, rectosigmoid colon)	13
Rectum	6
Large intestine with unspecified localization	7
Histological grade	
Grade 1	4
Grade 2	18
Grade 3	10
Unknown	7

and the histological staging was evaluated by experienced pathologist in line with TNM classification using the recommendation according WHO and IUCC [23, 24]. The histotype features associated with the MMR-deficient MSI CRC were based on the presence of TILs and the peritumoral lymphoid reaction. The presence of TILs and the peritumoral lymphoid reaction ("Crohn's-like" or lichenoid) was evaluated as positive or negative [25, 26]. The TILs were evaluated in 10 or more high-power fields and scored as present when there were five or more intraepithelial lymphocytes in at least one high-power field (40×) (Fig. 1a). The peritumoral lymphocytic reaction was examined in two forms as "Crohn's-like" or "lichenoid" reaction. The "Crohn's-like" reaction was assessed as positive (Fig. 1b) in cases when four and more nodular lymphoid aggregates/follicles were determined in low-power field (4×) laying beyond the advancing edge of the tumor within the subserosa or mesenteric fat [25]. Lichenoid reaction (Fig. 1c) was defined as a conspicuous lichenoid banding of lymphocytes found just beyond the advancing edge of the tumor [26]. The presence of at least one of the two examinated features was considered to be a positive peritumoral lymphocytic reaction. Figure 1d shows negative result for TILs and peritumoral lymphocytic reaction. The tumor was classified as mucinous carcinoma when at least 50% of the carcinoma showed mucin secretion. For statistical evaluation, the *G*-test was applied and *p* value less than 0.05 was considered as significant.

DNA preparation and MSI determination

DNA was extracted from FFPE sections after deparaffinization using commercially available kit blackPREP FFPE DNA Kit (Analytik Jena, Germany) according the manufacturer instructions as described previously [27]. The concentration of DNA was measured on the Qubit® 2.0 Fluorometer (Invitrogen, USA). For the MSI analysis, the samples were diluted on the concentration of 1–2 ng/µl and stored at − 20 °C. The MSI was determined using the MSI Analysis System Version 1.2 (Promega, Madison, WI, USA) kit according to the manufacturer's protocol (Kašubová et al. [22]) with a pentaplex polymerase chain reaction using five quasimonomorphic mononucleotide markers (NR-27, NR-21, NR-24, BAT-25 and BAT-26) and two pentanucleotide markers (PENTA C and PENTA D). The MSI was evaluated by fragment analysis on the ABI 3500 (Applied Biosystems, USA) with GeneMapper software (Applied Biosystems, USA). As MSI-high (MSI-H) were evaluated samples with two or more instable markers (Fig. 2), samples with one instable marker were evaluated as MSI-low (MSI-L), MSS and non-tumor samples had all marker stable.

POLE ED primer design and single-tube nested PCR

The PCR primers were designed in the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) in order to amplify four exons coding for the ED domain of the *POLE* gene (Table 2). Each primer pair was optimized

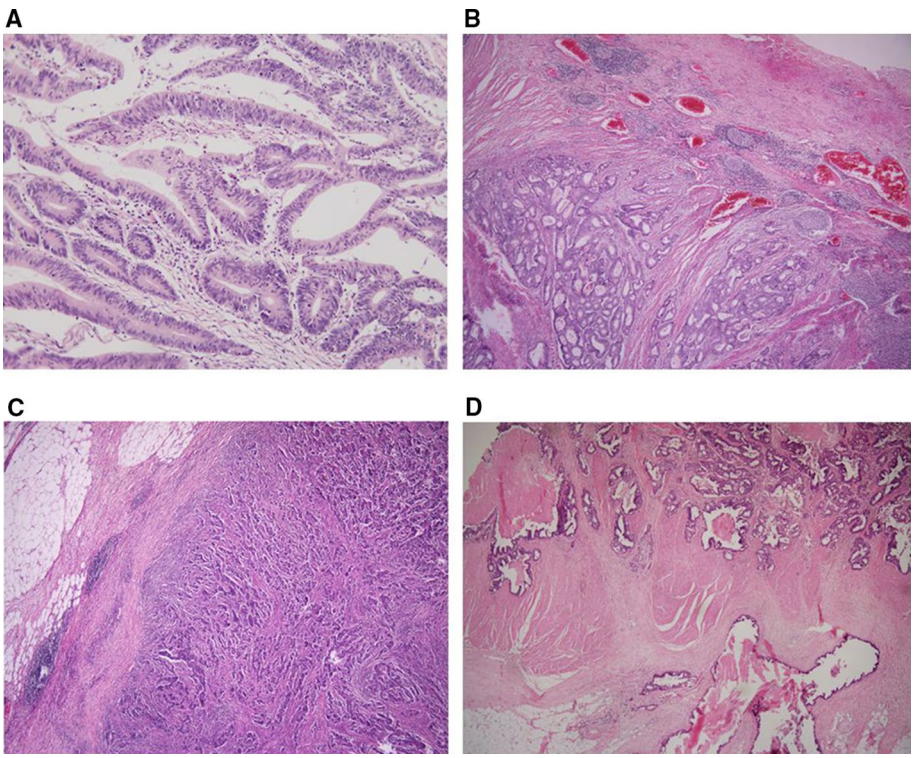


Fig. 1 HE-stained FFPE section from colorectal adenocarcinoma with **a** positive TILs, HE magnification (20×), **b** "Crohn's-like" reaction with numerous lymphoid follicles in subserous fat beyond the adenocarcinoma, HE magnification (4×), **c** Lichenoid reaction with continuous band of lymphocytes just beyond the advancing edge of the tumor in the subserosa, HE magnification (4×), **d** negative TILs and peritumoral lymphoid reaction, HE magnification (4×)

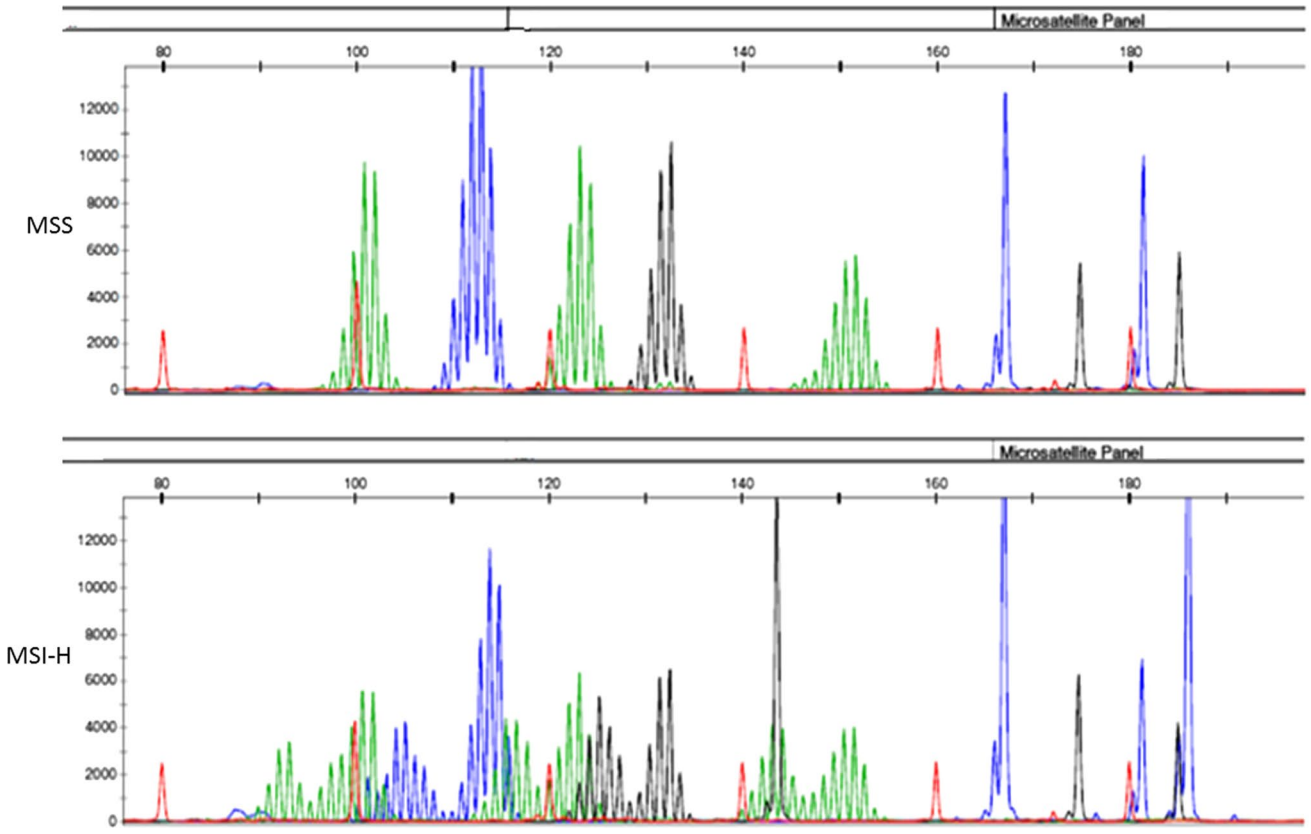


Fig. 2 Results of the fragment analysis of five quasimonomorphic mononucleotide markers and two pentanucleotide control markers showing microsatellite stable non-tumor (MSS) and microsatellite instable (MSI-H) result from the same patient

Table 2 DNA sequences and annealing temperatures of external and internal PCR primers

External primers	Sequence	T_A (°C)
POLE_ex9_extF	ACCAGAGGGAGGTAGAGCAGG	64
POLE_ex9_extR	GGTACAGCTGGAGGTCGGAAC	
POLE_ex11_extF	ATAAGGGCCAACATGAGGCTGC	60
POLE_ex11_extR	CTCCTAAGTCGACATGGGAAGC	
POLE_ex13_extF	TTTGCCAGTTCTCAGGGGTTC	64
POLE_ex13_extR	CCGGAGACACAGCCTGCTG	
POLE_ex14_extF	CCTGGGCTCTTGATTTTTGATGG	60
POLE_ex14_extR	CTGACGTCACACACCAGAATTC	
Internal primers	Sequence	T_A (°C)
POLE_ex9_intF	TGTTCAGGGAGGCCTAATGG	58
POLE_ex9_intR	CAGATGCTGCTGTAGTATGG	
POLE_ex11_intF	ACTTTGGGAGAGGAATTTGGAA	58
POLE_ex11_intR	AACGCCCTCCCTCTCAAATG	
POLE_ex13_intF	CATCCTGGCTTCTGTTCTC	56
POLE_ex13_intR	AGCGGGCTGGCATACATG	
POLE_ex14_intF	TCTCTGGCGTTCTCTCCTC	56
POLE_ex14_intR	CCTCCATTCAGCTCCAGTG	

in a separate PCR assay. The external and internal primers were used in a single tube until the optimal PCR conditions were set. The PCR reaction was performed using a 400 µM dNTPs, 5 × GoTaq Flexi Buffer, 2.5 mM MgCl2, 0.2 µM–0.4 µM external and internal forward and reverse primers, 2U of GoTaq® DNA polymerase (Promega, Madison, WI, USA) and 100 ng of DNA per reaction in a total volume of 25 µl. The amplification started with activation of Taq polymerase at 95 °C for 2 min. The first stage of the temperature program included 15 cycles with denaturation at 95 °C for 15 s, annealing at 64° C, respectively, at 60° C for 30 s (Table 2, external primers), an extension at 72 °C for 1 min. The second stage of the amplification consisted of 30 cycles with denaturation at 95 °C for 15 s, annealing at 58 °C, 56 °C, respectively, 54 °C for 30 s (Table 2, internal primers) and extension at 72 °C for 1 min. The last step was the final extension at 72° C for 5 min and cooling the products to 15° C.PCR products were evaluated by agarose electrophoresis containing GelRed reagent (Biotium, Hayward, CA, USA) and visualized using a UV lamp.

Sanger sequencing of PCR products and evaluation

The amplicons were purified with NucleoSpin Gel and PCR Clean-up (Macherey–Nagel, Duren, Germany) and evaluated by electrophoresis in a 1.75% agarose gel. Sanger sequencing was performed using the BigDye® Terminator v1.1 kit Cycle Sequencing (Applied Biosystems, Foster City, CA, USA) with internal forward primer F or reverse primer R (Table 2), and SigmaSpin post-reaction clean-up columns (Sigma-Aldrich, St. Louis, USA) were used for the second purification. The samples were analyzed on 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with Sequencing Analysis and SeqScape (Applied Biosystems, Foster City, CA, USA.) as reported previously [28] and compared to the reference sample (ENST00000320574.9). For the pathogenicity assessment of the mutation, in silico prediction tool, MutationTaster was used (http://www.mutationtaster.org/).

Results

EOCRC patients with MSI show higher infiltration with TIL

The mean age of the patients with EOCRC ($n = 39$) was 44.2 years with median about 46 years at diagnosis, four patients were MSI-H and 34 were MSS. From five patients with MSI-H, four exhibit the presence of TILs; in one case, we missed the data. On the contrary, only four patients with MSS have been proved as positive for TILs, 15 were negative; in 15 cases, the results were not available ($p = 0.004$). For peritumoral lymphoid reaction, one patient with MSI-H was negative and three were positive. Patients with MSS were mainly negative (11) or the data were no available (16), only seven were positive for peritumoral lymphoid reaction ($p = 0.205$) (Table 3).

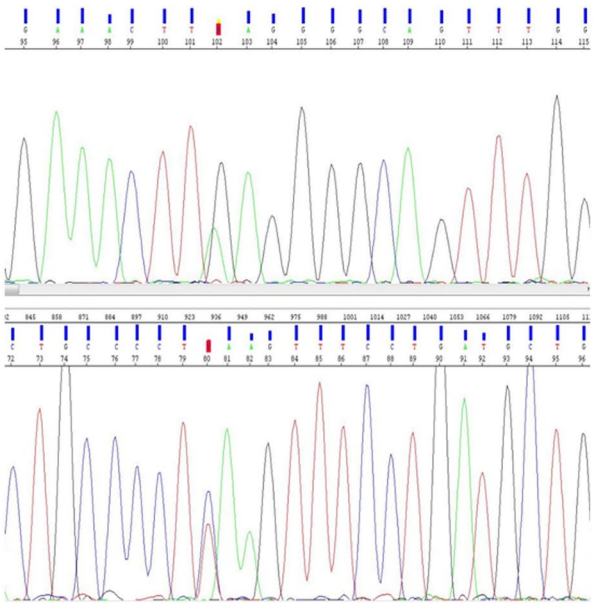
Detection of rare silent variant in exon 9 of the POLE gene

The primers were designed so that they sequenced the coding parts of exons 9, 11, 13 and 14, with adjacent intronic sequences. In our cohort, we identified within the intron 13 a common SNP rs4883555G_A, the frequency of allele G was 0.64 and A 0.36. According to the dSNP is this a common SNP with the ancestral allele G and MAF 0.42 as reported by 1000 Genome project.

In the coding sequence, we detect neither heterozygous variants for SNPs nor recurrent pathogenic mutations in proofreading domain of the protein. In one female patient diagnosed at age 48 with rectal adenocarcinoma with mucinous elements staged pT3pN2pM1, we have found a silent variant within the exon 9 NM_006231.3 c.849 C > T, NP_00622.2 p.Leu283 = recorded in dSNP as rs1232888774 with MAF = 0.00002 according to the database TOPMed (https://www.nhlbiwgs.org/). We confirmed these mutations with sequencing in both directions with reverse and forward primer (Fig. 3, left). According to the in silico prediction tool, MutationTaster is this mutation disease causing, probably deleterious and could be involved into splice site change (Fig. 3, right). In this case, the patient was MSS, and TILs and peritumoral lymphoid reaction were negative. The non-tumor tissue or peripheral blood was not available for mutation testing.

Table 3 Presence of TILs and peritumoral lymphoid reaction in patients with EOCRC (see the text)

	MSI ($n = 5$)	MSS ($n = 34$)	p value
TILs pos.	4	4	$p = 0.004$
TILs neg.	0	15	
Unknown	1	15	
Peritumoral lymphoid reaction pos.	3	7	$p = 0.206$
Peritumoral lymphoid reaction neg	1	11	
Unknown	1	16	



Prediction **disease causing**

Summary

- protein features (might be) affected
- splice site changes

analysed issue	analysis result
name of alteration	no title
alteration (phys. location)	chr12:133253192G>A show variant in all transcripts IGV
HGNC symbol	POLE
Ensembl transcript ID	ENST00000320574
Genbank transcript ID	NM_006231
UniProt peptide	Q07864
alteration type	single base exchange
alteration region	CDS
DNA changes	c.849C>T cDNA.893C>T g.160196C>T
AA changes	no AA changes

http://www.mutationtaster.org/cgi-bin/MutationTaster/MutationTaster69.cgi

Fig. 3 Sanger sequencing electropherogram of the exon 9 of the *POLE* gene with forward and reverse primer depicting the nucleotide substitution c.849 C > T, which results in silent mutation in codon p.L283 = (CTC > CTT) (left). MutationTaster interpretation of the variant (right)

Discussion

We have analyzed 39 patients with EOCRC representing a subset of patients with the disease onset as earlier as at age 52 from the cohort of 270 patients with CRC, in order to identify recurrent or new *POLE* gene mutations within the ED. We have implemented a sensitive single-tube nested PCR with subsequent Sanger sequencing to find also mutations present with low allele frequencies. In the intron 13, we found a common SNP rs4883555 whose frequency in dSNP for the minor allele is 0.42 as stated by 1000 genomes project (http://1000genomes.org). The frequency in our cohort corresponds to 0.36 which classified this SNP as a common one. Other authors have reported this SNP with similar frequency of 0.36 in Spanish cohort of patients with EOCRC [29].

In our cohort, we were not able to find already published recurrent pathogenic somatic mutations such as P286R that have been published to occur more frequent in younger patients or in patients with EOCRC without family history [12, 17]. The most common germline mutation L242 V was also absent in our cohort. A study that focused on genes causing hereditary colorectal cancer also reported none of these as recurrent known mutations in 59 patients with EOCRC and MSS, and 13 patients with EOCRC and MSI [29].

We have detected a very rare allele registered in dSNP as rs1232888774 that was found in the TOPMed project (https://www.nhlbiwgs.org/) with the frequency of 0.0002 as a germline variant and was not previously associated with CRC. Because we could not analyze the non-tumor tissue or DNA extracted from peripheral blood of this patient, we cannot exactly say if this variant is a germline or somatic one. We and other observed that both, the wild-type and the rare variant of the *POLE* gene, were present in tissues from patients diagnosed with CRC, and it was reported that the presence of one mutated allele can lead to the ultra-mutator phenotype [12, 13, 29]. According to the mutation pathogenicity prediction tool MutationTaster, this SNV was interpreted as probably deleterious having impact on splicing; however, functional study is necessary to confirm this prediction.

POLE-mutated tumor exhibits high number of TILs and neoantigen load, particularly increased CD8 + lymphocytes, cytotoxic T cell markers and effector cytokines, making them candidates for checkpoint inhibitor therapy [12, 14, 16]. In the study of Domingo et al. [12], it was shown that patients with *POLE* mutations in ED display enhanced immune response; however, the transcriptional signature was different when compared with consensus molecular classification 1 (CMS1) that is typical for MSI-H CRC. Despite of the high mutation burden, the authors concluded that *POLE*-mutated CRC are different from MSI CRC concerning the transcriptional signature [12]. In our case, we have used histotype features associated with MMR to assess the presence of TILs and the peritumoral lymphoid reaction. In our small subset of patients, the presence of TILs was associated with MSI-H as expected ($p = 0.004$). The patient with the variant NM_006231.3 c.849 C > T and MSS exhibits both histotype parameters as negative; therefore, we need

to finish functional study in order to find out if this variant really impairs the polymerase ε proofreading function with the impact on the mutation load and the immune response. Recently, it was reported that two of three patients mutated in *POLE* gene do not respond to checkpoint inhibitor therapy and had shown different PD-1 expressing CD + 8 TILs and TILs with Th1 phenotype in the TME that could predict response to these therapies [30], and therefore, not only the high mutation load itself is sufficient for lymphocyte infiltration of the tumor and response to the immunotherapy. Also mutations in genes that are involved into antigen presentation such as *B2 M* and *HLA* can have influence on TILs and a large-scale genomic analysis of MSI-H colorectal cancer demonstrated that hypermutated cancer frequently undergo and immunoediting processes allowing them to escape immune control despite of high mutational load [31]. Therefore, the role of *B2 M* mutations and their involvement into immune response in both MSS and MSI colorectal cancers should be studied more comprehensively in the future.

Conclusions

The increased frequency of *POLE*-mutated tumors among patients with EOCRC raises the possibility that this group of patients could benefit from the screening for mutations independently of familial history, because somatic events are possibly more common in this group as germline mutations. Moreover, the germline variants can be detected in the cancer tissue under certain circumstances [12, 13, 29]. Although in our case, the final confirmation of the pathogenicity remains to be elucidated by a functional study, the low allele frequency about 0.00002 and in silico prediction result showing the possible involvement into splicing are particular indicators that detected rare variant that can be involved into EOCRC pathogenesis. In the time of precise medicine, it is important to develop screening strategies also for less common conditions such as EOCRC in sense to predict tailored therapy for younger patients suffering from CRC that harbor mutations in the *POLE* gene.

Funding This study was supported by the Biomedical center Martin (ITMS 26220220187), which is co-financed from EU sources, by Government of Russian Federation, Grant 08-08, and by the projects of the Slovak Research and development Agency APVV Grant APVV-16-0066, and VEGA Grants 1/0199/17 and 1/0380/18.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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