ORIGINAL ARTICLE

Targeted next generation sequencing screening of Lynch syndrome in Tunisian population

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Abstract

A high colorectal cancer (CRC) incidence is observed in Tunisia, with a relatively high proportion of patients developing CRC before the age of 40. While this suggests a genetic susceptibility, only a few Tunisian Lynch Syndrome families have been described. In this study we aimed to identify the underlying genetic cause in 32 patients with early onset CRC and/or a positive family history. Of twenty-four patients' tumor or biopsies could be analyzed with immunohistochemical staining to detect loss of expression of one of the MMR proteins. Ten tumors showed loss of expression, of which one tumor was from a patient where a germline pathogenic *MSH2* variant was detected previously with Sanger sequencing. Next generation sequencing of the MMR, *POLE* and *POLD1* genes was performed in leukocyte and tumor DNA of the remaining nine patients, as well as in two patients with MMR-proficient tumors, but with severe family history. In six of 11 patients a germline variant was detected in *MLH1* (n = 5) or *MSH2* (n = 1). Two of six patients were from the same family and both were found to carry a novel in-frame *MLH1* deletion, predicted to affect MLH1 function. All *MLH1* variant carriers had loss of heterozygosity with retention of the variant in the tumors, while a somatic pathogenic variant was detected in the patient with the germline *MSH2* variant.

Keywords Lynch syndrome · Tumor · Immunohistochemical staining · DNA mismatch repair genes · MMR panel

Introduction

Lynch syndrome (LS;[MIM] 120435), is an autosomal dominant disease with early onset of colorectal cancer (CRC) and other extracolonic cancers including endometrium-, stomach-, small intestine-, hepatobiliary tract-, urinary

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tract-, ovarian-, brain- and skin cancer [1]. LS is caused by heterozygous pathogenic germline variants in the DNA mismatch repair (MMR) genes: *MLH1* (MIM 120436)*, MSH2* (MIM 609309), *MSH6* (MIM 600678) and *PMS2* (MIM 600259) [2, 3]. The majority of causative germline variants are located in *MLH1* and *MSH2* (50% and 40% respectively), while 10% of variants are located in *MSH6* and *PMS2* [4]. Additionally, deletions in *EPCAM* (MIM 185535) have been described to result in hypermethylation of the *MSH2* promoter and subsequent silencing of *MSH2* [5].

The inactivation of the MMR pathway can also be caused by somatic events, such as *MLH1* promoter hypermethylation or bi-allelic somatic mutations in MMR genes [6–8]. *MLH1* promoter hypermethylation is present in 15% of sporadic CRCs and is more frequent in older patients without family history of CRC [9].

Few studies of CRC in Tunisia are reported and little is known about the underlying genetics [10]. The incidence of CRC in Tunisia in 2009 was about 12.4/100.000 inhabitants [11]. A higher CRC rate has been described in European countries such as Danish population where CRC has an incidence of 160/100.000 [12]. However, since a consistent nationwide screening program is lacking, this number is likely underestimated and is predicted to more than double in 2024 [11]. Furthermore, a relatively high proportion of patients develop tumors before the age of 40 years (14.2%), suggesting a genetic susceptibility [10].

Until now, only few Tunisian LS families were described. A 2011 study sequencing the MMR genes in 31 unrelated Tunisian families suspected of LS based on age of onset or positive family history, found germline MMR variants in 52.6% of male and 8.3% of female patients tested, leaving a large fraction of patients unexplained [10]. Previously, bi-allelic somatic inactivation of the MMR genes and germline variants in other CRC susceptibility genes, as *POLE* and *POLD1,* have been described in suspected Lynch Syndrome patients, but this has never been tested in a Tunisian population and prevalence of these variants in Tunisia remains unknown [8, 13]. The aim of the current study was to identify the underlying genetic cause in 24 Tunisian index patients with suspected LS, early-onset CRC and/or a positive family history. To this end, next-generation sequencing of the MMR genes and *POLE* and *POLD1* was performed.

Materials and methods

Cohort description

In this study, 32 patients suspected for Lynch Syndrome from 29 different Tunisian families were investigated. All patients developed colorectal cancer, with an average onset age of 43.2 years (range 19–75 years, Table 1). Leukocyte- and

Table 1 Cohort overview

	Number of patients (%)
Gender	
Male	18 (56)
Female	14 (44)
Age of onset	43.2 years
Family history	
FDR + LS	23 (72)
SDR + LS	18 (56)
No family history	1 (3)
Unknown	4 (12.5)
Amsterdam II	26 (81.25)

FDR + Ls first-degree relative with LS-associated tumors, *SDR + Ls* second-degree relative with LS-associated tumors

tumor DNA was available for 24 patients (75%) and tumor DNA of these patients was isolated either from biopsies (n = 6) or formalin-fixed paraffin-embedded (FFPE, n = 18) tissue blocks. Of the remaining cases (n = 8) FFPE and biopsies were not available. Patients were recruited between 2009 and 2017 from University Hospital Farhat Hached—Sousse (n = 12), University Hospital Sahloul—Sousse (n = 6), University Hospital Fatouma Bourguiba—Monastir (n = 2), University Hospital Tahar Maamouri—Nabeul (n = 1) and private gastro-intestinal doctors (n = 3).

Available information concerning sex, age of onset, site and size of the tumor and CRC familial history were verified. The majority of patients tested (72%) had a first-degree relative with a Lynch-associated tumor and 26 of 32 patients were from families fulfilling Amsterdam II criteria (Table 1).

Sanger sequencing

First, 18 patients were pre-screened with Sanger Sequencing for variants in *MLH1* and *MSH2* (Supplementary Table 1). Sequencing was done as previously described [14]. Primers are shown in Supplementary Table 2.

Immunohistochemical staining

Immunohistochemical staining (IHC) of the four MMR proteins (MLH1, MSH2, MSH6 and PMS2) was performed on available FFPE tissue blocks or paraffin-embedded biopsies (n = 24, Supplementary Table 1), using a previously described standard protocol [15].

Next-generation sequencing

Total DNA was isolated from all FFPE-tissue blocks or biopsies [16] that showed expression loss of at least one

of the MMR proteins by immunohistochemistry (n = 11) with the tissue preparation system [17]. DNA concentration was measured with the Qubit® 3.0 fluorometer. Of all patients with MMR-deficient tumors, leukocyte (n = 11) and tumoral DNA (n = 10) was sequenced with the Ion Proton System (Life Technologies, Carlsbad, CA, USA) using a custom MMR panel [8]. Libraries were prepared with Ion AmpliSeq™ Library Kit 2.0 according to the manufacturer's protocol. The Proton sequencer generated unaligned BAM which were mapped against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters (https://github.com/iontorrent/TS).

All variants were filtered on minor allele frequency (< 0.05), variant frequency (> 10%) and coverage (> 50x). The following Genbank reference sequences were used: NM_000249.3 for *MLH1*, NM_000251.2 for *MSH2*, NM_000179.2 for *MSH6*, NM_000535.5 for *PMS2*, NM_006231.2 for *POLE* and NM_001256849.1 for *POLD1*. In silico analysis of all variants was done using Mutation Taster [17], Polyphen 2 [18] and UniProt [19].

Variants classification was done according to the InSiGHT MMR gene variant classification criteria (https://www.insight-group.org). Variants were classified as: Class 5: pathogenic, Class 4: likely pathogenic, Class 3: Variant of uncertain significance (VUS), Class 2: Likely not pathogenic/little clinical significance, Class 1: Not pathogenic/low clinical significance.

Results

Immunohistochemical staining was performed on sections from all available biopsies (n = 6) and formalin-fixed paraffin-embedded (FFPE) tissue blocks (n = 18). Thirteen tumors/biopsies (54%) showed positive staining of all four MMR proteins, whereas 10 tumors (42%) showed loss of expression of MLH1/PMS2 (n = 6) or MSH2/MSH6 (n = 4). In the final patient staining results were inconclusive due to poor quality of the tumor tissue.

Sanger sequencing was firstly performed on 18 patients, with a pre-screening for some germline exonic variants in *MLH1* and *MSH2*. Two patients were found to carry a germline variant, either a nonsense variant (*MSH2* c.1413dupA, patient TUN4) or a variant of uncertain significance (*MLH1* c.218T > C, patient TUN13), while the rest tested negative for germline variants.

Tumor DNA was isolated from 9 of 10 MMR-deficient tumors, as well as two MMR-proficient tumors (TUN3 and TUN7) with MMR-proficient tumors, but with severe family history (Supplemental Fig. 1). NGS of the four MMR genes, *POLE* and *POLD1* was performed on tumor- and normal DNA of all 11 patients.

With NGS, a germline variant was detected in the MMR gene that showed expression loss with IHC (Table 2) in six patients. Patient TUN25 and TUN26 are related (Fig. 1), and in both patients a germline in-frame *MLH1* deletion was detected. The remaining four detected variants were a nonsense variant (n = 1) and non-synonymous missense variants of uncertain significance (VUS, n = 3). All missense variants showed loss of heterozygosity (LOH) in the tumor, while the patient with the nonsense variant (TUN28), was found to carry a second somatic nonsense variant (*MSH2* c.2557G > T) explaining the tumor phenotype. Patient TUN19 and TUN30 were found to carry the same *MLH1* VUS (c.218 T > C), however, patients were not related. In the two tested patients with MMR- proficient tumors (TUN3 and TUN7), no germline or somatic

**Table 2** Variants detected with Sanger and NGS

Patient	IHC	Tumour	Gene	Variant	Amino acid alteration	Class of the variant	%	
							N	T
TUN4	MSH2-/MSH6-	CRC49	*MSH2*	c.1413dupA[a]	p.(Pro472Thrfs*4)	5	NA	
TUN13	MLH1-/PMS2-	CRC29	*MLH1*	c.218T > C[a]	p.(Leu73Pro)	3	48	90[b]
TUN19	MLH1-/PMS2-	CRC39	*MLH1*	c.1918C > A	p. (Pro640Thr)	3	44	66[b]
TUN25	MLH1-/PMS2-	CRC42	*MLH1*	c.1940_1951delTGCCCCCTTTGG	p.(Val647_Leu650del)	3	48	72[b]
TUN26	MLH1-/PMS2-	CRC44	*MLH1*	c.1940_1951delTGCCCCCTTTGG	p.(Val647_Leu650del)	3	50	85[b]
TUN28	MSH2-/MSH6-	CRC45	*MSH2*	c.1255C > T	p.(Gln419*)	5	48	50
				c.2557G > T	p.(Glu853*)	4	–	29
TUN30	MLH1-/PMS2-	CRC55	*MLH1*	c.1918C > A	p.(Pro640Thr)	3	50	70[b]

IHC proteins that showed loss of expression, *Tumor* tumor type followed by age of onset, % variant allele frequency in leukocyte DNA (N) or tumor DNA (T)

*Stop codon

[a]Variant detected with Sanger sequencing

[b]Loss of heterozygosity was seen in the tumor
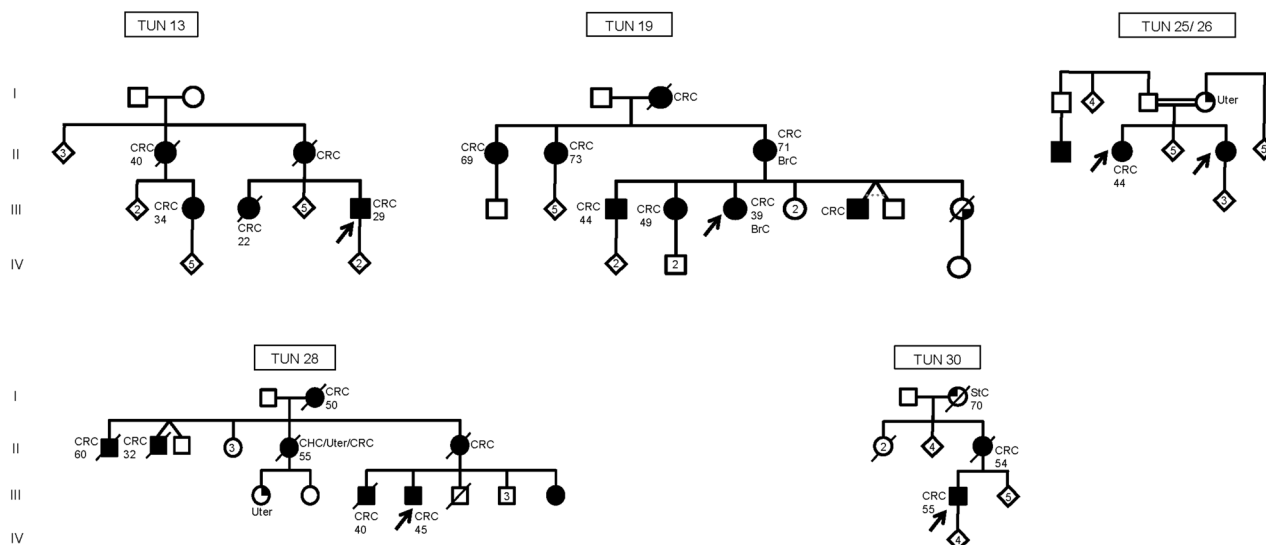
Fig. 1 Pedigrees of MMR variant carriers. Pedigrees of patients with germline MMR variants. *CRC* colorectal cancer, *BrC* breast cancer, *Uter* uterine cancer, *StC* stomach cancer, *CHC* hepatocarcinoma. Age at diagnosis is mentioned with the type of the cancer; circles represent females; squares represent males; diamonds represent undisclosed gender; cross striped individuals are deceased. Filled symbols represent affected family members (CRC), while the open symbols are unaffected family members black right upper corner presents family member with Uter cancer, black left upper corners presents family member with StC, black right lower corner presents family member with BrC. Index patients are indicated with the arrow

MMR variants were detected. Additionally, no *POLE* or *POLD1* exonuclease domain variants were found.

Discussion

The incidence of colorectal cancer (CRC) in Tunisia is increasing and is expected to rise to 39.3/100.000 patients in 2024 [11]. However, even though a high proportion (14.2%) of these CRC patients are under 40, indicating a possible genetic susceptibility, only a few Tunisian Lynch Syndrome (LS) families have been described [10].

In the current study, DNA from 32 patients from 29 families was collected to test for germline or somatic variant in the MMR genes, but also in *POLE* and *POLD1*. Pre-screening of leukocyte DNA of 18 patients only detected a pathogenic variant in one patient (TUN4) and a variant of uncertain significance (VUS) in a second patient (TUN13). The pathogenic variant (*MSH2* c.1413dupA), is a frameshift variant resulting in a premature stop codon in exon 9, previously described in multiple Lynch Syndrome families. The patient carrying this mutation (TUN4) was a male with a 10 cm sigmoid tumor appearing at the age of 49 years (Supplemental Table 1). The sigmoid tumor of patient TUN13 showed loss of MSH2 and MSH6 staining with immunohistochemistry (IHC). No material was available of other family members.

Immunohistochemical staining of MLH1, MSH2, MSH6 and PMS2 in 24 tumors and biopsies showed loss of expression in ten patients (from nine families). Next generation sequencing (NGS) was performed on leukocyte- and tumor DNA for nine of ten patients (except TUN4), with MMR-deficient tumors, as well as two patients TUN3 and TUN7, (Supplemental Fig. 1) with MMR-proficient tumors but with a strong family history of CRC. Patient TUN4 was not screened with NGS, since a pathogenic variant was already detected with Sanger in the gene that showed immunohistochemical loss of expression (Fig. 2).

With NGS, six germline variants were detected in the gene that showed immunohistochemical expression loss. One variant was detected before with Sanger (*MLH1* c.218T > C). A *MLH1* c.1918C > A variant was found in two obviously unrelated patients (TUN19 and TUN30) who were not pre-screened with Sanger Sequencing. While both variants are still classified as class 3 (variant of uncertain significance) by the LOVD database according to InSiGHT classifications, previous studies have shown partial loss of MMR for both variants [20]. In silico analysis of the *MLH1* c.1918C > A predicted a damaging effect, through affecting the interaction with the Exonuclease I (EXO1) protein, and a previous study shows the variant results in only 53% MMR activity [20]. The *MLH1* c.218T > C variant is also predicted to affect function, and a previous study shows 34–66% loss of MMR function [21]. The patients' affected sister (CRC22, Fig. 1), was tested for the variant and was also found to be a carrier, supporting possible pathogenicity of the variant.

Patients TUN25 and TUN26 (two sisters) were found to carry the same novel *MLH1* c.1940_1951delTGCCCC
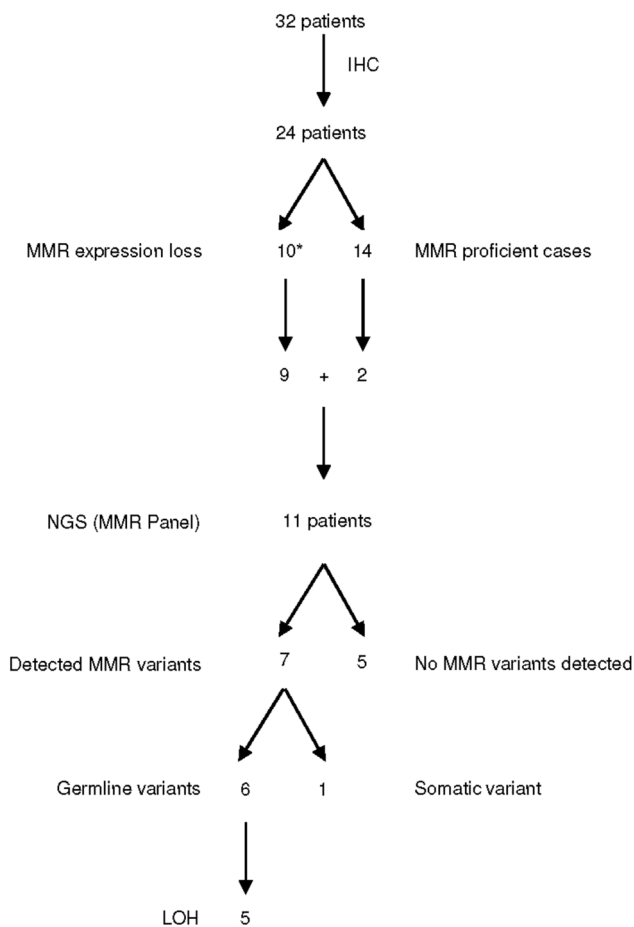
Fig. 2 This figure shows the patient selection, methods chosen and results found. *No *MLH1* hypermethylation test performed

CTTTGG in-frame deletion. Both showed loss of heterozygosity with retention of the variant in the tumor. In silico analysis predicts loss of the MLH1-EXO1 interaction. Known missense variants within this region (c.1942C > T, c.1943C > T) have been described to be pathogenic and have been shown to result in altered protein localization [22, 23]. Patient TUN28 was found to carry a germline *MSH2* c.1255C > T nonsense variant, with an additional somatic *MSH2* c.2557G > T nonsense variant in the tumor. This is a known pathogenic variant, previously described in a Dutch population [24].Unexpectedly, the patients affect sister (CRC34), did not carry this variant.

In three of 10 patients (TUN8, TUN11 and TUN15) with MMR-deficient tumors no germline or somatic MMR, *POLE* or *POLD1* variant was detected. Interestingly, the average age of onset of these three patients was 26,6 years (19–37 years), and two of three were from Amsterdam II positive families. This strongly suggests that there is an underlying genetic cause in these families, which has currently not been detected.

In this study, two assays were performed on somatic tumor cells sections or DNA to perform IHC and NGS. They represent robust and complemented techniques for a direct suspicion and confirmation of the right diagnosis. Together they allow a major gain in time and cost.

A recent study, published by Hampel H et al. in 2018, showed that NGS sequencing of tumor DNA can replace all current standard tests, including those used universally for tumor screening in LS [25].

In 13 index patients no loss of expression was detected in any of the MMR genes. While Lynch Syndrome is unlikely in these patients, an underlying genetic cause cannot be excluded. Four of 13 families fulfilled Amsterdam II criteria, and could therefore be classified as familial colorectal type X (FCCTX) families, for which currently no one underlying gene defect is found [26–28]. Previous studies found variants in *SEMA4A*, *ACVRLK3* and *SETD6* that could possibly explain a small fraction of these patients, but the majority of FCCTX patients remain unexplained [29]. Furthermore, germline variants in the exonuclease domain of *POLE* and *POLD1* have been described to cause polymerase associated polyposis syndrome, in which patients predispose to early onset colorectal cancer, often with polyps [30–32]. While these tumors are often microsatellite stable, secondary MMR variants resulting in MMR loss also have been described in PPAP (Polymerase Proofreading-Associated Polyposis) patients, and the phenotype remains broad [8, 13]. For this reason in this study, three families with MMR-proficient tumors, but with a severe family history (Supplemental Table 1) were tested for variants in these genes, but no genetic variant was detected. Implementation of a large gene panel or whole exome sequencing could possibly find the genetic cause in these patients [33].

In summary, with a custom NGS panel and Sanger sequencing we were able to detect a germline MMR variant in seven of ten patients with MMR-deficient tumors. In 13 patients no MMR-deficiency was found in the tumor and the underlying genetic cause remains unknown.

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