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ORIGINAL ARTICLE

Germline genomic profiling of patients with early-onset colorectal cancer

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Background: The incidence of early-onset colorectal cancer (EO-CRC) is rising. While most cases of EO-CRC are sporadic, the prevalence of hereditary cancer predisposition syndromes remains a subject of debate. Moreover, genes not traditionally associated with EO-CRC development are rarely included in germline testing panels.

Patients and methods: Germline profiling data of patients with EO-CRC presenting to our clinics were collected at two Italian university institutions: IRCCS San Raffaele Scientific Institute and Grande Ospedale Metropolitano Niguarda. Multigene germline profiling analysis was carried out using next-generation sequencing and multiplex ligation probe amplification. Associations between germline alterations and clinicopathological variables were analyzed.

Results: A total of 130 patients with EO-CRC were screened. The median age at EO-CRC diagnosis was 42 years (range 22-49 years). Germline pathogenic or likely pathogenic variants (PVs/LPVs) associated with hereditary cancer predisposition syndromes were identified in 23 (18%) patients, while germline variants of unknown significance were found in 47 (36%) patients. No alterations in high-penetrance genes associated with cancer susceptibility were observed in 67 (52%) patients. No patients with microsatellite stable *BRAF*-mutant ($n = 5$) or signet ring cell CRC ($n = 2$) exhibited germline PVs/LPVs. No clinicopathological features were significantly enriched in hereditary compared with sporadic EO-CRC. Germline PVs in *FLCN* and *SDHAF2* were identified in two patients with EO-CRC.

Conclusions: While most EO-CRC cases are sporadic, approximately one-fifth arises within the context of hereditary cancer predisposition syndromes. As *FLCN* and *SDH* are not currently included in the current guidelines for EO-CRC, PVs/LPVs in these genes may be underestimated. To better understand their significance, we recommend including their assessment in all patients with EO-CRC.

Key words: *FLCN* gene, *SDHAF2* gene, *BRAF*, early-onset colorectal cancer, signet ring cell carcinoma, genetics

INTRODUCTION

Colorectal cancer (CRC) is the third most common and third most lethal cancer worldwide.[1] While CRC incidence and mortality rates are declining in the overall population, a result largely attributable to the growing implementation of widespread screening campaigns and the significant strides made in both immunological and molecularly targeted therapies,[2] the incidence has been steadily rising in adults younger than 50 years of age since the early 1990s.[3-6] Notably, the rate of this increase is most acute among individuals younger than 35 years of age.[4] Despite current screening recommendations being based on age rather than biological rationale, CRCs arising in young individuals are commonly referred to as early-onset CRC (EO-CRC).[7,8] This epidemiological shift has been notably pronounced in the United States, where EO-CRC has already become the leading cause of cancer-related mortality among men aged 20-49 years.[4,9] Similar trends have been observed worldwide, including in European countries.[5,6,10] Moreover, epidemiological models suggest that by 2030, approximately one-third of all new CRC diagnoses will occur in individuals younger than 50 years of age.[4,7] Consequently, the rise of EO-CRCs has emerged as a significant unmet need in both clinical setting and cancer research.[11]

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Although the majority of EO-CRCs are sporadic, the prevalence of hereditary cancer predisposition syndromes is reportedly higher among patients younger than 50 years of age compared with older patients with CRC.[7,12] Indeed, 9.0%-26.4% of patients with EO-CRCs have an underlying pathogenic alteration, and this proportion may be even higher in those younger than 35 years of age.[13] The vast majority of these hereditary EO-CRCs occur in the context of Lynch syndrome (LS) or familial adenomatous polyposis (FAP).[12] However, a spectrum of other germline pathogenic variants (PVs) in genes distinct from those implicated in LS and FAP can also confer an increased risk of earlier-than-expected CRC development.[14] The prevalence of non-LS hereditary predisposition ranges from 2.3% to 26.4%. A critical unresolved question, however, is whether EO-CRCs stemming from non-LS and non-FAP germline alterations exhibit unique clinicopathological features that could support the development of targeted and personalized diagnostic panels. Moreover, the possibility of identifying a subset of EO-CRC cases in which germline genetic analysis may be unnecessary based on specific clinicopathological characteristics warrants further investigation. Indeed, in our recent guidelines, we recommended that all patients with EO-CRC must be screened for hereditary cancer syndromes regardless from oncological family history, and disease clinicopathological features.[14]

We conducted an observational retrospective study to review findings from extended germline profiling in a consecutively collected cohort of patients with EO-CRC screened at two Italian institutions. We analyzed the clinicopathological and molecular characteristics of this cohort to identify potential indicators for refining hereditary cancer syndrome screening strategies, ultimately aiming to improve the management of patients with EO-CRC.

PATIENTS AND METHODS

We retrospectively collected and reviewed results from hereditary cancer predisposition syndrome screening conducted via germline profiling at Grande Ospedale Metropolitano Niguarda and Università degli Studi di Milano, and at IRCCS San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan, Italy. All patients under 50 years of age who presented to our clinics with a diagnosis of EO-CRC and previously unknown germline status underwent genetic counseling and germline multigene panel testing, in accordance with the recent Delphi Initiative for Early-Onset Colorectal Cancer (DIRECt) international guidelines.[14] In conducting our panels, we adhered to the recommendations outlined in the DIRECt international guidelines.[14] All patients provided informed consent for germline profiling after receiving comprehensive information about genetic testing. The multigene germline panels used are detailed in the Supplementary Materials, available at https://doi.org/10.1016/j.esmogo.2025.100182. At both institutions, extended next-generation sequencing (NGS) panels and multiplex ligation-dependent probe amplification were carried out on genes known to be associated with CRC (please refer to Supplementary Materials, available at https://doi.org/10.1016/j.esmogo.2025.100182). Additional genes outside the standard panel were analyzed based on clinical suspicion arising from each patient's family history and comorbidities. Data on PVs and likely pathogenic variants (LPVs) and variants of unknown significance (VUS) were collected. The identified germline variants were categorized as PVs or LPVs according to the American College of Medical Genetics and Genomics guidelines.[15] A family history of CRC in first- and second-degree relatives, along with a history of cancer at other sites, was also collected as part of the genetic counseling process.

In addition to germline test results, epidemiologic and clinicopathological features, standard-of-care molecular data, and treatment outcomes were retrieved through an extensive electronic chart review at both institutions.

The study was conducted in accordance with the World Medical Association Declaration of Helsinki and was reviewed and approved by the Institutional Review Boards of IRCCS San Raffaele Scientific Institute (Protocol BIO-GASTRO/2011) and Grande Ospedale Metropolitano Niguarda [IANG-CRC - PROGETTO IANG-CRC (frrb.it)], for which dedicated written informed consent was obtained.

Descriptive statistics were conducted, and germline findings were compared with clinicopathological features using Fisher's exact or the chi-square test.

RESULTS

From January 2018 to February 2025, all patients who attended our dedicated outpatient clinics with a diagnosis of EO-CRC received multigene germline testing, resulting in a total of 130 consecutive patients with EO-CRC. Patients' characteristics are summarized in Table 1. A total of 69 (53%) patients were male, while 61 (47%) were female. The median age at diagnosis was 42 years (range 22-49 years). Although the majority of patients (119/130, 92%) were Italian, 11/130 (8%) originated from other countries. Specifically, 6/11 (55%) were from other European countries, 3/11 (27%) from South America, and the remaining 2/11 (18%) were from Africa.

Overall, the clinicopathological characteristics observed in our cohort were consistent with those typically expected in patients diagnosed with EO-CRC.[7,8] The majority of our cohort (96/130, 74%) had primary tumors in the rectum or left colon, and a significant proportion (27/130, 21%) displayed mucinous or signet ring cell features. In the subgroup of patients who developed metastasis, 12/44 (27%) had peritoneal or ovarian involvement. Finally, 99/130 (76%) had a family history of cancer, and 25/130 (19%) had a first-degree relative with CRC diagnosed at any age, with 12% (3/25) of them having a family history of EO-CRC.

We identified at least one germline PV or LPV in 23 patients (18%; Figure 1). Among these 23 cases, at least one PV was found in 20 (87%), while at least one LPV was identified in 4 (17%; Table 2). Moreover, at least one VUS was found in 47 out of 130 (36%) patients (Table 3), of whom 40 harbored only a VUS (30%) without any

Table 1. Clinicopathological features of early-onset colorectal cancer patients screened for germline alterations according to the identification of germline PVs/LPVs, VUS, or no alterations at all

Clinicopathological variable	Germline PV/LPV *n* = 23 (18%)	Germline VUS *n* = 40 (30%)	No germline variants *n* = 67 (52%)	Overall population *n* = 130 (100%)
Hospital, *n* (%)				
San Raffaele, MI, IT	20 (87)	31 (78)	55 (82)	106 (81)
Niguarda, MI, IT	3 (13)	9 (22)	12 (18)	24 (19)
Age (years) at diagnosis, median (range)	40 (23-49)	42 (22-49)	42 (25-49)	42 (22-49)
Sex, *n* (%)				
Male	12 (54)	21 (50)	36 (54)	69 (53)
Female	11 (46)	19 (50)	31 (46)	61 (47)
Country of origin, *n* (%)				
Italy	20 (86)	36 (90)	63 (94)	119 (94)
Other countries	3 (14)	4 (10)	4 (6)	11 (8)
Inflammatory bowel disease, *n* (%)				
Yes	0 (0)	1 (3)	2 (3)	3 (1)
No	23 (100)	38 (95)	65 (97)	127 (98)
Unknown	0 (0)	1 (2)	0 (0)	1 (1)
Cancer family history, *n* (%)				
Yes	21 (91)	26 (65)	52 (78)	99 (76)
No	2 (9)	14 (35)	15 (22)	31 (24)
Any CRC family history, *n* (%)				
Yes	12 (52)	10 (25)	30 (45)	53 (40)
No	11 (48)	30 (75)	36 (54)	77 (59)
Unknown	0 (0)	0 (0)	1 (1)	1 (1)
CRC in first-degree relatives, *n* (%)				
Yes	8 (35)	6 (15)	11 (17)	25 (19)
No	15 (65)	34 (85)	55 (81)	104 (80)
Unknown	0 (0)	0 (0)	1 (2)	1 (1)
CRC in second-degree relatives, *n* (%)				
Yes	9 (39)	7 (17)	25 (37)	41 (31)
No	14 (61)	33 (83)	41 (62)	88 (68)
Unknown	0 (0)	0 (0)	1 (1)	1 (1)
Patient diagnosed with cancers other than CRC, *n* (%)				
Yes	3 (14)	5 (12)	7 (11)	15 (12)
No	20 (86)	35 (88)	60 (89)	115 (88)
Other polyps at colonoscopy, *n* (%)				
Yes	4 (17)	4 (10)	7 (11)	15 (12)
No	16 (70)	33 (83)	54 (80)	103 (79)
Unknown	3 (13)	3 (7)	6 (9)	12 (9)
Sidedness, *n* (%)				
Rectal or left-sided[a]	17 (74)	29 (73)	50 (75)	96 (74)
Right-sided[b]	6 (26)	11 (27)	17 (25)	34 (26)
Histology, *n* (%)				
Adenocarcinoma NOS	16 (70)	30 (75)	56 (84)	102 (78)
Mucinous carcinoma	7 (30)	10 (25)	8 (12)	25 (19)
SRCC	0 (0)	0 (0)	2 (3)	2 (2)
Other	0 (0)	0 (0)	1 (1)	1 (1)
Grading, *n* (%)				
G1	0 (0)	1 (3)	2 (3)	3 (2)
G2	13 (56)	18 (45)	35 (52)	66 (52)
G3	3 (14)	11 (27)	14 (21)	28 (21)
Unknown	7 (30)	10 (25)	16 (24)	33 (25)
Stage at diagnosis, *n* (%)				
I	4 (17)	7 (17)	9 (14)	20 (15)
II	8 (35)	5 (12)	15 (22)	28 (21)
III	7 (31)	18 (45)	15 (22)	40 (32)
IV	4 (17)	9 (23)	21 (32)	34 (26)
Unknown	0 (0)	1 (3)	7 (10)	8 (6)
Somatic *RAS* mutations, *n* (%)				
Yes	5 (23)	5 (12)	11 (16)	21 (16)
No	1 (5)	4 (10)	12 (18)	17 (14)
Unknown	16 (73)	31 (78)	44 (66)	91 (70)
Somatic *BRAF* mutations, *n* (%)				
Yes	0 (0)	1 (2)	4 (6)	5 (4)
No	6 (26)	10 (25)	18 (29)	34 (26)
Unknown	17 (74)	29 (73)	45 (65)	91 (70)
Somatic MMR status[c], *n* (%)				
MSS	12 (52)	24 (60)	39 (58)	75 (58)
MSI	4 (16)	6 (15)	8 (12)	18 (14)
Unknown	7 (32)	10 (25)	20 (30)	37 (28)

Continued

Table 1. Continued

Clinicopathological variable	Germline PV/LPV *n* = 23 (18%)	Germline VUS *n* = 40 (30%)	No germline variants *n* = 67 (52%)	Overall population *n* = 130 (100%)
Metastatic development, *n* (%)				
Yes	6 (26)	11 (27)	27 (40)	44 (34)
No	8 (35)	12 (30)	21 (32)	41 (31)
Unknown	9 (39)	17 (43)	19 (28)	45 (35)
Sites of metastasis, *n* (%)[d]				
Liver	3 (50)	6 (54)	16 (59)	25 (57)
Lung	1 (17)	3 (27)	4 (15)	8 (18)
Nodes	1 (17)	4 (36)	6 (22)	11 (25)
Peritoneum ± ovary	2 (33)	5 (45)	5 (19)	12 (27)
Other sites	1 (17)	2 (18)	3 (11)	6 (14)
Number of lines for metastatic disease, median (range)	3 (1-3)	1 (1-3)	1 (1-6)	1 (1-6)

CRC, colorectal cancer; IT, Italy; LPV, likely pathogenic variant; MI, Milan; MMR, mismatch repair; MSI, microsatellite unstable; MSS, microsatellite stable; NOS, not otherwise specified; PV, pathogenic variant; SRCC, signet ring cell carcinoma; VUS, variant of unknown significance.
[a]Located in the splenic flexure, descending colon and sigmoid colon, or rectum.
[b]Located in the caecum, ascending colon, liver flexure, and transverse colon.
[c]As assessed by immunohistochemistry.
[d]Percentage here refers to patients who develop metastatic disease: 6, 11, 25, and 42 among those with germline pathogenic variants, variants of unknown significance, no alterations, and considering the overall population, respectively.

concomitant PV/LPV. No germline alterations were detected in the remaining 67 patients diagnosed with EO-CRC (52%; Figure 1). Germline pathogenetic alterations affecting mismatch repair (MMR) genes were found in 48% of all patients with at least one PV/LPV (11/23). Additionally, 26% of EO-CRCs with at least one germline PV/LPVs in our cohort (6/23) had alterations in the *MUTYH* gene, including three homozygous and three heterozygous carriers. Among other clinically actionable high-penetrance genes associated with CRC, 5/23 (22%) patients had at least one PV/LPVs in DNA damage response (DDR) genes such as *ATM*, *BRCA2* ($n = 2$), *BRCA1*, and *CHEK2* (Table 2). Two patients carried two germline LPV/PVs: one patient with microsatellite stable (MSS) EO-CRC presented with concomitant germline *ATM* and *CHEK2* PVs, while another patient with MSI EO-CRC had concomitant germline *MLH1* and *MUTYH* PVs (Table 2).

The remaining two were patients diagnosed with rare germline PVs in genes not commonly associated with early CRC development. One patient was found to harbor the *FLCN* c.1252delC (p. Leu418Trpfs*50) heterozygous variant, which is known to cause Birt—Hogg—Dubé syndrome. He had no family history of CRC, but his mother was diagnosed with ovarian cancer at age 40 years and a head and neck cancer at age 62 years (Figure 2). He was diagnosed with stage IV MSS *RAS/BRAF* wild-type rectal cancer. On circulating tumor DNA, NGS by FoundationOne Liquid CDx detected mutations in *APC R1114**, *ARAF S214F*, *ARID1A Q553fs70*, *PIK3R1 L570_D578del*, *FLCN L418fs50*, and *TP53 E286D*. No solid tumor NGS profiling was available. The patient received four lines of standard-of-care treatment, to which the tumor became progressively resistant, and the patient passed away 27 months after the initial diagnosis. Another patient had a germline *SDHAF2* c.261-2A>T heterozygous LPV. *SDHAF2* germline PV/LPVs cause hereditary paraganglioma—phaeochromocytoma syndrome, but it has been reported as a potential driver of EO-CRC. She denied a

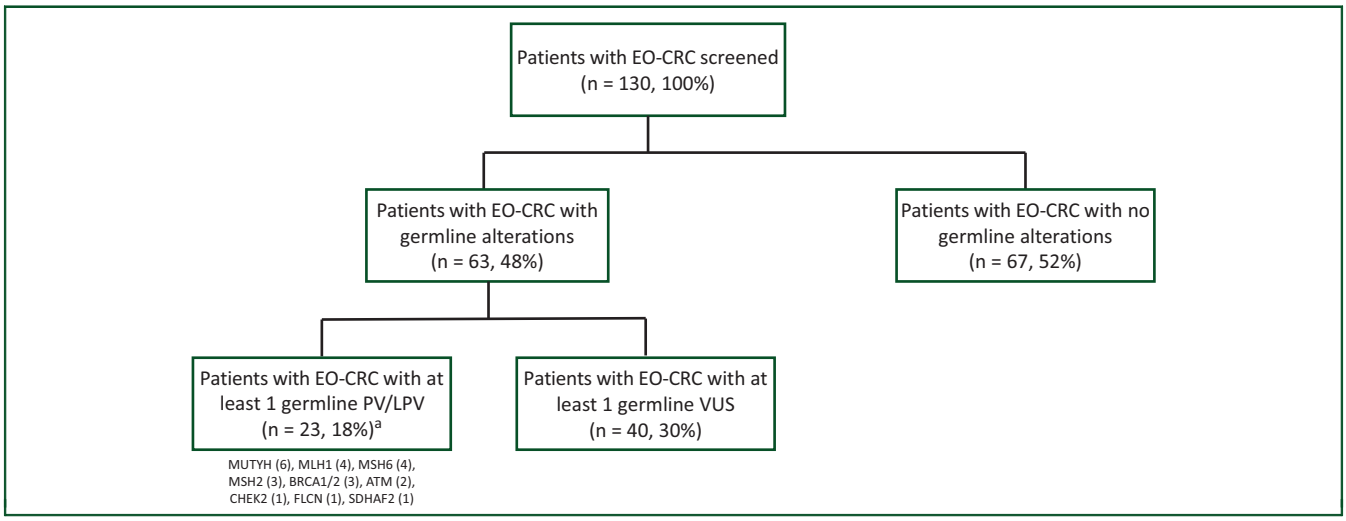

Figure 1. CONSORT diagram summarizing findings from our germline screening cohort of early-onset colorectal cancer.
EO-CRC, early-onset colorectal cancer; LPV, likely pathogenic variant; PV, pathogenic variant; VUS, variant of unknown significance.
[a]7 out of 22 (32%) had also at least one germline variant of unknown significance.

Table 2. Germline PVs/LPVs identified in our early-onset colorectal cancer cohort, according to somatic mismatch repair status as assessed by immunohistochemistry, and cancer familiarity

Gene	Mutation	Somatic MMR	Non-CRC familiarity	CRC familiarity[a]	Age (years)	Side	Stage	Histology	Pathogenicity
MUTHY	c. 1187G>A Homo.	MSS	Yes	No	29	L	4	NOS	PV
	c.734G>A Homo.	MSS	Yes	Yes	38	RE	3	NOS	PV
	c.1145G>A Homo.	N/A	Yes	Yes	43	RE	1	NOS	PV
	c.933+3A>C Hetero.	MSS	Yes	No	40	RE	4	MUC	PV
	c.1187G>A Hetero	N/A	No	No	48	RI	3	NOS	PV
MLH1	c.931_932delAA	N/A	No	Yes	23	L	2	NOS	LPV
	c.790+1G>A	N/A	Yes	Yes	37	L	2	NOS	PV
	c.677+3A>G	N/A	Yes	Yes	40	RI	2	NOS	PV
MSH6	c.679_680delAG	MSS	Yes	Yes	49	L	2	MUC	PV
	c.3261dup	MSS	Yes	Yes	46	L	2	MUC	PV
	c.2150_2153delTCAG	MSS	Yes	No	36	L	3	MUC	PV
	c.3261del (p.Phe1088Serfs*2)	MSI	No	Yes	48	RE	3	NOS	PV
MSH2	g.(47705466_47707826)_(47709946_?)	MSI	Yes	Yes	24	RI	1	MUC	LPV
	c.942+3A>T	MSI	Yes	Yes	24	RI	2	NOS	PV
	c.2131C>T (p.Arg711X)	N/A	Yes	Yes	37	RI	2	NOS	PV
MUTHY/MLH1	c.1187G>A/ c.1609C>T	MSI	Yes	No	48	L	2	MUC	PV/PV
BRCA2	c.7007G>A	MSS	Yes	No	42	RI	1	NOS	PV
	c.2979G>A	N/A	Yes	Yes	37	RI	1	NOS	PV
ATM/CHEK2	c.2502dupA Hetero./ c.1169A>T Hetero.	MSS	Yes	No	37	L	3	NOS	PV/LPV
ATM	c.5763-2A>T	MSS	Yes	No	47	RE	4	NOS	PV
BRCA1	c.190T>C p.(Cys64Arg)	MSS	Yes	No	32	L	3	MUC	PV
FLCN	c.1252delC Heterozigous	MSS	Yes	No	46	RE	4	NOS	PV
SDHAF2	c.261-2A>T	MSS	Yes	No	42	RE	3	NOS	LPV

CRC, colorectal cancer; L, left-sided; LPV, likely pathogenic variant; MMR, mismatch repair; MSI, microsatellite unstable; MSS, microsatellite stable; MUC, mucinous; N/A, not applicable; NOS, adenocarcinoma not otherwise specified; PV, pathogenic variant; RE, rectal; RI, right sided.
[a]At least one first- or second-degree relative diagnosed with colorectal cancer.

family history of CRC but had a second-degree relative with breast cancer diagnosed after the age of 60 years and another second-degree relative with ovarian cancer at the age of 80 years on the paternal side. On the maternal side, she reported a second-degree relative with brain cancer at the age of 62 years and another second-degree relative with anal cancer at the age of 55 years. The patient was initially diagnosed with stage III MSS rectal cancer at the age of 42 years. Seventeen months after the initial diagnosis, the patient experienced a local relapse with nodal and peritoneal involvement. After three lines of metastatic treatment, to which the tumor became progressively resistant, the patient passed away 34 months after the initial diagnosis.

We analyzed clinicopathological and molecular features based on the presence or absence of PV/LPVs and VUS, with results summarized in Table 1. No clinicopathological variable demonstrated a statistically significant association with an increased likelihood of detecting germline PVs/LPVs. A family history of CRC was not predictive of germline PV/LPVs, as only 12/23 (52%) patients with germline PVs/LPVs reported a family history of CRC in any first- or second-degree relatives. Five of the six metastatic EO-CRC cases diagnosed in patients harboring germline PVs/LPVs were *RAS* mutant, while none were *BRAF* mutant. Notably, we identified three somatic MSS EO-CRC cases, based on immunohistochemistry, in patients diagnosed with LS due to *MSH6* germline PV/LPVs (Table 2). Moreover, although a substantial majority of patients with at least one PV/LPV (21/23, 91%) reported a family history of cancer, those with PVs/LPVs in genes other than *MSH2*, *MSH6*, or *MLH1* (namely, *MUTYH*, *ATM*, *BRCA1*, *BRCA2*, *FLCN*, *SDAHF2*, *CHEK2*) were significantly less likely to have a family history of CRC (3/12, 25%; $P = 0.03$).

Among patients without PVs/LPVs or VUSs, none of those diagnosed with either *BRAF*-mutant MSS EO-CRC ($n = 5$; 4 with $BRAF^{V600E}$ and 1 $BRAF^{D594D}$) or MSS signet ring cell carcinoma (SRCC; $n = 2$) were found to harbor any germline PVs/LPVs. Notably, $BRAF^{V600E}$ is known to be mutually exclusive with LS. However, given the small sample size, further studies are warranted to determine whether the presence of such somatic features may help identify EO-CRC subgroups with a lower incidence of harboring hereditary cancer predisposition syndromes.

DISCUSSION

The incidence of EO-CRC is rising worldwide.[5,7,10] Although predominantly sporadic, up to 30% of patients with EO-CRC may have an underlying hereditary cancer predisposition syndrome.[12,16,17] This paper presents a consecutively collected cohort of patients diagnosed with EO-CRC who underwent germline cancer susceptibility testing as part of standard clinical care.

The 18% prevalence of germline PVs/LPVs in potentially actionable high-penetrance genes in our cohort highlights the importance of screening for hereditary cancer syndrome in patients diagnosed with EO-CRC and their at-risk relatives. In line with previous studies,[12,16,17] we strongly support the recommendation that all patients with EO-CRC undergo germline screening. Ideally, this should be carried out at the time of initial diagnosis, given its potential implications for both surgical decision making and systemic treatment planning.[18]

Table 3. VUS identified in 47 out of 130 (36%) patients from our early-onset colorectal cancer cohort, according to somatic mismatch repair status as assessed by immunohistochemistry, and cancer familiarity

Gene	Variant of unknown significance	Somatic mismatch repair status	Pathogenic variants
RAD51D	c.796C>T	MSS	None
MSH3 *MSH6* *ATM*	c.2886_2888delAAT c.805A>G c.5890A>G	MSS	None
BRCA1	c.4514 A>G	MSS	None
MSH6	c.733 A>T	MSS	None
CHEK2	c.1024G>A	MSS	None
PALB2	c.1241G>A	MSI	None
APC	c.4435G>T	MSS	None
MLH1	p.(Tyr126Asn)	MSS	None
MSH2	c.499G>C p. Asp167His	MSS	None
MSH6	p.(Leu1167Val)	MSS	None
POLE	p.(Arg47Trp)	MSS	None
APC *BMPR1A*	p.(Arg1069Gly) p.(Arg443Cys)	MSI	None
BRCA1	c.5365G>T	MSS	None
MSH6 *CDH1*	c.3019T>C c.2204C>A	MSS	None
PALB2 *CDK4*	c.3307G>A c.719G>A	MSS	None
CHEK2	c.470T>C - p.(Ile157Thr)	MSS	None
RAD51C	c.960G>C	MSS	None
APC	c.6473C>G p.(Pro2158Arg)	MSS	None
MLH1 *BRCA2*	c.1526_1528del p.(Leu509del) c.8971C>T p.(Arg2991Cys)	MSI	None
MSH3	p.(Pro1050Arg)	MSS	None
MLH3	c.818A>G p.(Tyr273Cys)	MSS	None
MSH3	p.Ile945Met	MSS	None
CHEK2	c.1060G>A p.(Asp354Asn)	Unknown	None
BRCA1 *TP53*	c.500A>G p.(Gln167Arg) c.677G>T p.(Cys226Phe)	Unknown	None
CDH1	c.88C>A p.(Pro30Thr)	MSI	None
CDH1	c.674T>C p.(Ile225Th)	MSS	None
POLE	c.2683G>A p.(Ala895Thr)	MSS	None
NBN	c.872A>G	MSS	None
MLH1	c.589-10_589-7del	Unknown	None
MSH6	p.(Ala342Gly)	Unknown	None
CDH1	c.1142A>G p.(Lys381Arg)	Unknown	None
CDH1	c.1774G>A p.(Ala592Thr)	Unknown	None
TP53	c.446C>T	Unknown	None
BMPR1A	c.418C>T p.(Pro140Ser)	MSS	None
MUTYH	c.1276C>T p.(Arg426Cys)	MSS	None
CHEK2 *STK11*	c.544C>A c.312G>C	Unknown	None
MSH6	c.11A>T p.(Gln4Leu)	Unknown	None
APC	p.(Leu1029Arg)	MSS	None
STK11	c.1225C>T (p.Arg409Trp)	MSI	None
CHEK2	c.565A>G p.(Ile232Val)	MSI	None
CDH1	c.2387G>A	Unknown	None
APC	c.8479G>A p.(Gly2827Arg)	MSS	None
MSH3	c.1777C>T p.Arg593Trp	MSS	None
BRCA1	c.2503C>T p.(His835Tyr)	MSS	None
PMS2	c.857A/G, p.(Asp286Gly)	MSS	None
RAD51C	c.211A/T, p.(Asn71Tyr) heterozygous	MSS	None
MSH2	c.435T>G p.Ile145Met	MSS	ATM/CHEK2
MLH1	*p.(Tyr126Asn)*	*MSS*	*MUTYH*
RAD51C	*c.960G>C*	*MSS*	*BRCA2*
MLH3	*c.818A>G p.(Tyr273Cys)*	*MSS*	*MUTYH*
MSH3	*p.Ile945Met*	*MSS*	*ATM*
APC	*p.(Leu1029Arg)*	*MSS*	*BRCA1*
MSH3	*c.1777C>T p.Arg593Trp*	*MSS*	*SDHAF2*

MSI, microsatellite unstable; MSS, microsatellite stable; VUS, variant of unknown significance.

Our analysis of clinicopathological and molecular data from our EO-CRC patient cohort reveals findings that are broadly consistent with previously published data from multiple institutions and countries.[12,16,19] The proportion of EO-CRC cases linked to hereditary cancer predisposition syndromes ranges between 10% and 33%, with higher rates observed among patients younger than 35 years of age.[12,16,19] In our Northern Italy-based cohort, approximately one-fifth of EO-CRC diagnoses were associated with hereditary cancer syndromes, corroborating these prior observations. The identification of germline PVs/LPVs has significant implications for both screening strategies and the therapeutic decision making for patients and their families. Accordingly, we reaffirm our recommendation that all patients with EO-CRC should undergo comprehensive germline profiling, regardless of their family history of cancer or somatic tumor profiling results.[14]

With regard to gene selection for NGS-based germline panels in patients with EO-CRC, the first DIRECt guidelines on EO-CRC clinical management recently proposed a comprehensive multistep approach. This begins with a core set of genes definitively associated with CRC, followed by the inclusion of additional genes that are reasonably prevalent and clinically actionable.[14] Our prospective cohort study showed that an upfront expansion of the initial germline panel to include more genes driving hereditary cancer syndromes potentially associated with CRC could offer substantial clinical advantages for patients and their relatives. In this context, beyond the well-established involvement of *MMR* and *MUTYH* genes, and building on previous reports,[20] DDR genes accounted for 22% of all germline PVs/LPVs in our cohort. Notably, one patient harbored concomitant *ATM* and *CHEK2* PVs in the setting of a strong family history of non-CRC cancers. This finding is clinically significant, as alterations in DNA repair pathways are increasingly being explored as therapeutic targets in metastatic CRC within ongoing clinical trials. These trials involve various DDR inhibitors, including, but not limited to, poly(ADP-ribose) polymerase inhibitors.[21,22] Moreover, the identification of a PV/LPV in DDR genes, such as *ATM*, *BRCA1/2*, and *CHEK2*, enables tailored preventive surveillance and/or prophylactic surgeries as part of dedicated screening campaigns, benefitting both patients and their relatives.[23-27]

In our cohort, neither a family history of cancer in general nor a family history of CRC significantly predicted the identification of germline PV/LPV. We observed only a numerical trend toward enrichment for cancer- or CRC-specific family history among patients in whom a hereditary cancer predisposition syndrome was identified, possibly due to the small sample size. Notably, patients with EO-CRC and a germline PV/LPV in non-MMR genes (i.e. DDR genes, *FLCN*, and *SDHAF2*) were significantly less likely to report a CRC-specific family history. This suggests that broadening germline screening beyond typical CRC-related genes could help identify additional hereditary cancer predisposition

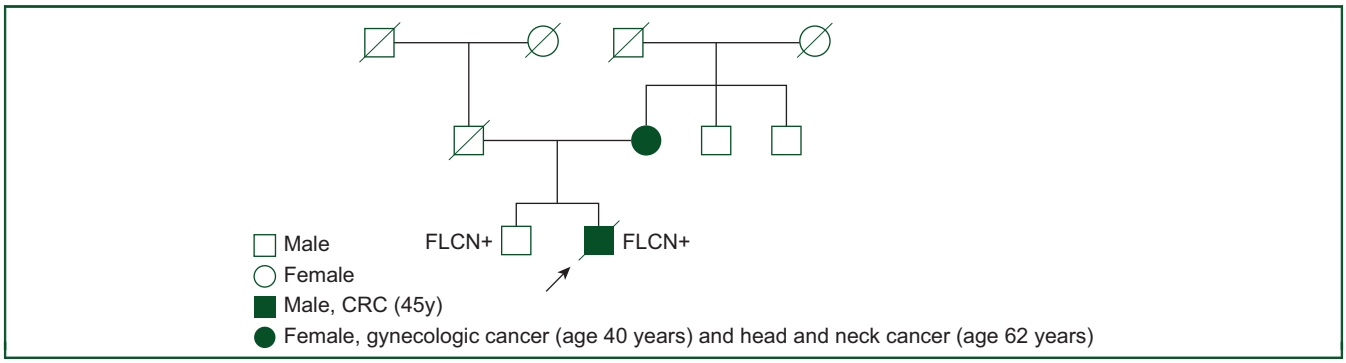

Figure 2. Family tree of the patient with early-onset colorectal cancer (CRC) diagnosed with Birt–Hogg–Dubé syndrome following the identification of the germline *FLCN c.1252delC p.Leu418Trpfs*50* heterozygous variant.

syndromes not commonly associated with early CRC development. Of note, while DDR genes were already included in the panel discussed in the guidelines,[14] we identified two patients with EO-CRC harboring germline mutations in *FLCN* and *SDHAF2*—these two genes were not included in the DIRECt germline panel.[14]

The *FLCN* gene is physiologically expressed in multiple tissues, and its protein product has been associated with the regulation of cell growth and survival, metabolism, and cell adhesion.[28] *FLCN* interacts with numerous signaling pathways, including the protein kinase B/mechanistic target of rapamycin pathway.[29] Although the underlying mechanisms are not yet fully elucidated, dysregulation of *FLCN* contributes to an increased risk of tumorigenesis in patients with Birt–Hogg–Dubé syndrome.[30] Notably, *FLCN* germline PVs in patients with Birt–Hogg–Dubé syndrome have been reported to increase the risk of EO-CRC, despite *FLCN* not being among the genes typically screened for germline alterations in patients with EO-CRC.[31] Patients with Birt–Hogg–Dubé syndrome exhibit a moderate but significant increase in the risk of developing CRC (5.1% versus 1.5%; $P = 0.0068$), with up to 35% of them fulfilling the revised Bethesda criteria.[31] However, the prevalence of germline *FLCN* mutations in EO-CRC remains to be systematically investigated.

SDHAF2 germline PVs cause hereditary paraganglioma–phaeochromocytoma syndrome but have also been associated with EO-CRC.[32-34] SDH genes encode the various subunits of succinate dehydrogenase, a key respiratory enzyme involved in the Krebs cycle and the electron transport chain.[35] Particularly, *SDHAF2* encodes SDH complex assembly factor 2 (SDHAF2), which is essential for the flavination of the SDHA protein and enzyme activity.[36] *SDH* PVs typically predispose individuals to the development of pheochromocytoma and paraganglioma, gastrointestinal stromal tumors, renal cell carcinoma, and pituitary adenomas. However, over the past two decades, the spectrum of SDH-related tumors has expanded to also include CRC.[32-34] Although a direct mechanistic association between *SDHAF2* PVs and CRC is still lacking, the expression of SDHAF2 at the intestinal level makes a potential link plausible. This assumption is further supported by the fact that SDHAF2 is required for SDHA activity, and *SDHA* PVs have been associated with CRC development. Thus, if confirmed in other cohorts and international studies systematically assessing their prevalence in patients with EO-CRC, both *FLCN* and *SDAHF2* genes could be included in standard diagnostic NGS panels used to screen for hereditary cancer predisposition syndromes in EO-CRC.

We believe that the publications describing hereditary cancer syndrome screening in prospective EO-CRC cohorts are important for progressively defining the most comprehensive NGS panels to be used for patients with EO-CRC. This is even more important in the context of VUS, which we identified in 36% of patients with EO-CRC. Based on our findings, we recommend increasing the reporting of not only PV/LPVs but also VUSs identified in EO-CRCs, to help redefine their potential pathological role in the future. This could be crucial for both the scientific literature and the clinical management of patients with EO-CRC.

Upon reviewing patients with EO-CRC without any germline variants, we observed that all patients whose CRC was either *BRAF* mutant or characterized by SRCC features also lacked germline PVs/LPVs. However, this is expected in the context of $BRAF^{V600E}$ mutations and LS. Given the small sample size of these CRC subsets in our cohort, further data are needed to determine whether patients with EO-CRC with these features have a lower incidence of hereditary cancer syndromes.

Our study has several limitations. First, although our cohort of 130 patients provides valuable insights into EO-CRC, the sample size remains relatively limited. Second, the gene coverage of the NGS panels used at the two participating institutions was not entirely uniform. Third, the predominantly Italian origin of our cohort, with only 8% of patients from other countries, potentially limits the generalizability of our findings to other ethnicities and global populations. Fourth, because somatic NGS profiling of CRC is not yet recommended by international guidelines,[37,38] somatic NGS data were not available for direct comparison with germline findings.

In conclusion, our prospective cohort study, conducted within everyday clinical practice, confirms that approximately one-fifth of EO-CRC cases arise from germline PVs and fall within the framework of hereditary cancer syndromes, consistent with the DIRECt guidelines. Although a family history of cancer did not prove to be a reliable predictor of germline PV, patients with EO-CRC and germline PV in non-MMR genes were significantly less likely

to report a family history of CRC. The prevalence of hereditary cancer syndromes in EO-CRC may increase with the systematic reporting of VUS and their potential future reclassification. Finally, this study also provides evidence of germline alterations in *FLCN* and *SDHAF2*, warranting further investigation in future EO-CRC germline paneling studies. Finally, we propose larger germline screening studies focused on $BRAF^{V600E}$ mutant or SRCC EO-CRC cohorts to evaluate their potential association with hereditary cancer syndromes.

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DISCLOSURE

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