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RESEARCH ARTICLE

Clinical and molecular detection of inherited colorectal cancers in northeast Italy

A first prospective study of incidence of Lynch syndrome and *MUTYH*-related colorectal cancer in Italy

E. Urso · M. Agostini · S. Pucciarelli · M. Rugge · R. Bertorelle · I. Maretto · C. Bedin · E. D'Angelo · C. Mescoli · M. Zorzi · A. Viel · G. Bruttocao · B. Ferraro · F. Erroi · P. Contin · G. L. De Salvo · D. Nitti

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Abstract The reported incidence of hereditary colorectal cancers (CRCs) is widely variable. The principal aim of the study was to prospectively evaluate the incidence of familial CRCs in a region of northern Italy using a standardized method. Consecutive CRC patients were prospectively enrolled from October 2002 to December 2003. Patients underwent a structured family history, the microsatellite instability (MSI) test and a screen for *MUTYH* mutations. Following family history patients were classified as belonging to high, moderate and mild risk families. Immunohistochemistry for MLH1, MSH2, MSH6 and PMS2 proteins and investigation for *MLH1*/*MSH2* mutations, for *MLH1* promoter methylation and for the V600E hotspot *BRAF* mutation were performed in high MSI (MSI-H) cases. Of the 430 patients enrolled, 17 (4%) were high risk [4

Synopsis For evaluate prospectively the incidence of hereditary colorectal cancers (CRC) in a region of Italy, 430 patients were enrolled. A strong family history of CRC was present in 4% of cases; incidence of MLH1/MSH2 or MUTHY mutations was 1.3%.

E. Urso (✉) · M. Agostini · S. Pucciarelli · I. Maretto · C. Bedin · P. Contin · D. Nitti
Clinica Chirurgica 2nd, Dipartimento Scienze Chirurgiche, Oncologiche e Gastroenterologiche,
University of Padova, Padua, Italy,
Policlinico, VI piano, Via Giustiniani, 2,
35128 Padua, Italy
e-mail: edl@unipd.it

M. Agostini
Department of Nanomedicine,
The Methodist Hospital Research Institute,
Houston, TX, USA

M. Agostini · C. Bedin · E. D'Angelo
Istituto Ricerca Pediatrica, Città della Speranza,
Padua, Italy

M. Rugge · C. Mescoli
Servizio di Anatomia Patologica, University of Padova,
Padua, Italy

R. Bertorelle
Immunologia e Diagnostica Molecolare Oncologica, IOV-IRCCS,
Padua, Italy

M. Zorzi
Registro Tumori del Veneto, IOV-IRCCS,
Padua, Italy

A. Viel
Centro Riferimento Oncologico, IRCCS, CRO,
Aviano, Italy

G. Bruttocao
Chirurgia Geriatrica, University of Padova,
Padua, Italy

B. Ferraro
Chirurgia Generale, Ospedale San Antonio, ULSS 16,
Padua, Italy

hereditary non-polyposis colorectal cancer (HNPCC), 12 suspected HNPCC and 1 MUTYH-associated adenomatous polyposis coli (MAP)], 53 moderate risk and 360 mild risk cases. The MSI test was performed on 393 tumours, and 46 (12%) of them showed MSI-H. In these patients, one *MLH1* pathogenetic mutations and two *MSH2* pathogenetic mutations were found. Thirty-two (70%) MSI-H cases demonstrated *MLH1* methylation and/or *BRAF* mutation: None of them showed *MLH1*/*MSH2* mutation. Two biallelic germline MUTYH mutations were found, one with clinical features of MAP. A strong family history of CRC was present in 4% of the enrolled cases; incidence of *MLH1*/*MSH2* or *MUTHY* mutations was 1.3% and of MSI-H phenotype was 12%. *MLH1* methylation and *BRAF* mutation can exclude 70% of MSI-H cases from gene sequencing.

Keywords Lynch syndrome · Incidence of familial CRCs · Genetics screening

Introduction

One of the main goals of the public health programs is to recognize risk factors for common and severe diseases. Colorectal cancer (CRC) is one of the most frequent tumours worldwide, and familial inheritance for this tumour is a well-known risk factor. The most frequent hereditary CRC syndromes are the classic familial adenomatous polyposis (FAP), the attenuated familial adenomatous polyposis related to *APC* mutation (AFAP), the *MUTYH*-associated polyposis (MAP) and the hereditary non-polyposis colorectal cancer (HNPCC) syndrome. HNPCC is the most frequent one, and surveillance programs for at risk subjects have been found to improve survival [1]. Both clinical and molecular criteria are used to define this syndrome. The clinical criteria are those reported as Amsterdam I and II criteria [2, 3], whereas the molecular basis of HNPCC is a mutation in one of mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6* and *PMS2*, the first two being responsible for approximately 90% of the mutations found [4]. Sensitivity and specificity of Amsterdam I and II criteria in detecting mutations in one of MMR genes are 60% and 70% and 78% and 61%, respectively [5]. Since MMR gene sequencing is time- and cost-consuming, screening tests to select the best candidates for genetic tests have been proposed. They include microsatellite instability (MSI) testing and an immunohistochemical (IHC) assay for MMR proteins on tumour specimens. MSI is the hallmark of HNPCC-related cancer, being present in more than 90% of these tumours [6]; IHC assay for the MMR proteins on the tumour specimen has been reported to be a pre-screening test as effective as MSI [7].

Compared to HNPCC, FAP syndrome is less common and shows a pathognomonic phenotype with hundreds of colonic polyps at colonoscopy. FAP is related to germline deficiency of the APC gene [8]. Mutation carriers of a known FAP family are usually included in colonoscopic surveillance programs and a prophylactic colectomy before the development of invasive carcinoma. CRC is therefore an unusual presentation in APC carriers, unless they have a de novo mutation. Compared to the classic FAP, the AFAP shows an attenuated phenotype with fewer colonic polyps (from 10 to 100), a later age of onset, unusual extra-colonic manifestations and frequent involvement of the proximal colon at diagnosis [9]. The role of biallelic *MUTYH* mutations in the development of polyposis cases (thus defining the MAP) has recently emerged [10–12]. Even if rarely severe polyposis could be related to *MUTYH* mutations, MAP phenotype is very similar to the AFAP one [10]. *MUTYH* is involved in the base excision repair pathway that repairs DNA after free-radical damage [11]. Only the biallelic deficiency of *MUTYH*, which is inherited in a recessive manner [10, 11], has been shown to be pathogenetic, whereas no definitive data are available on the biological significance of the *MUTYH* monoallelic deficiency [10].

The ambiguity in defining HNPCC (clinical versus molecular diagnosis) and AFAP/MAP polyposis, the difficulty in distinguishing germline disease from phenocopies, the geographic variability and the lack of population-based studies explain the variability on the reported incidence of hereditary CRC. In addition, in Italy, the incidence of hereditary CRC is missing.

The main purpose of the study was therefore to prospectively evaluate, using clinical and molecular criteria, the incidence of familial CRC in the Padua area (northeast Italy). With the term of familial CRC, we mean either cases with strong family history of CRC (e.g. families fulfilling Amsterdam criteria), with or without a disease causing mutation found, either cases with pathogenetic mutation on *MMR*, *APC* or *MUTYH* genes, with or without clinical or familial criteria of the disease fulfilled. The ancillary end-points of the study were to verify the reliability of the family history as a mean to diagnosing HNPCC, to define the incidence of MSI and of the *MUTYH* mutations in the CRCs and to evaluate the clinical and molecular characteristics of tumours with MSI and without *MMR* gene deficiency.

F. Erroi
Clinica Chirurgica 3, University of Padova,
Padua, Italy

G. L. De Salvo
Servizio di Epidemiologia Clinica, IOV-IRCCS,
Padua, Italy

E. D'Angelo
Clinica Chirurgica 2nd, University of Padova,
Padova, Italy

Present Address:
P. Contin
Surgical Department, Heidelberg University,
Heidelberg, Germany

Patients and methods

This study was carried out in the local health district of Padua, a mostly urbanised area in the Veneto region (in northeast Italy). A significant portion of the population is older (20.4% are aged ≥65 years), and there are approximately 410,000 inhabitants of which 197,000 males and 213,000 are females.

Two studies were performed: The first one was retrospective and the second was prospective. In both studies, based on the clinical features and family history, the patients were subdivided into the following at-risk groups:

1. *High risk*: patients with FAP, defined as those with ≥100 adenomatous polyps at colonoscopy; patients with AFAP, defined as those with 10–99 colonic polyps and APC mutation; patients with *MUTYH*-related adenomatous polyposis coli (MAP), defined as patients with polyposis coli and biallelic mutation on *MUTHY* gene and patients with HNPCC and suspected HNPCC (s-HNPCC) as defined by the criteria of Amsterdam II [3] and Park [13], respectively
2. *Moderate risk*: patients with one first-degree relative with CRC before the age of 60 years or two second-degree relatives with CRC at any age
3. *Mild risk*: patients with no first- or second-degree relatives affected by CRC

Retrospective study

The principal aim of this study was to evaluate the ability of the family history, as routinely reported in clinical charts, to identify families at moderate/high risk for CRC. The form of family history available in clinical records is not standardized, and it is not the same in different surgical units.

One researcher of the Epidemiologic Unit of the Veneto Region Tumour Registry reviewed all clinical records of patients with CRC resident in the district of Padua between 2000 and 2001. Family history was classified as adequate or inadequate depending on whether the information was reliable enough to assign the patient to one of the risk groups; it has been recoded also if the hospitalization for CRC surgery was in the Padua district or outside our area. The patients were subdivided into risk groups as shown in the previous paragraph.

Prospective study

This part of the study was designed as a prospective hospital- (including all patients who underwent surgery for CRC at Padova Central Hospital) and population-based (including only patients resident in the Padua district) series and was approved by the Local Ethics Committee. The study was performed from November 1, 2002 to December 31, 2003 and included patients who underwent surgery in one of the eight surgical units of the Padova Central Hospital, signed the informed consent and had a histologically confirmed CRC. The patients were subdivided into risk groups as shown in the previous paragraph.

The following investigations were planned in all enrolled patients: a structured family history, an MSI test and a search for *MUTYH* mutations. Patients who showed MSI also underwent IHC for MLH1, MSH2 and MSH6 proteins and a screen for *MLH1* or *MSH2* mutations. The flow chart of this strategy is summarised in Fig. 1.

Family history

All patients were interviewed by a pool of three surgeons involved in the study, using a structured and standardized questionnaire dealing with malignancies, cancer location and age at onset of cancer among all known relatives. A genealogic tree was made for each patient. The presence and number of polyps at colonoscopy were also recorded.

DNA sample

DNA was extracted from fresh-frozen tumour tissues and from the EDTA-preserved blood samples with the use of standard methods (Qiamp Mini Kit, Qiagen, Hilden, Germany). The histologic features of the tumour were evaluated by analysing of the paraffin-embedded tissue block.

Microsatellite instability analysis

An MSI test was performed on paired tumour and normal tissue DNA samples using the National Cancer Institute panel of microsatellite markers (BAT26, BAT25, D2S123, D5S346 and D17S250) and an automatic ABI3130 DNA analyzer (Applera, Foster City, CA, USA). Tumours were scored as MSI-H (high instability), MSI-L (low instability) or MSS (stable), according to the Bethesda criteria [14, 15].

To improve the selection for MMR gene sequencing, all cases showing an MSI-H phenotype were also investigated for methylation of the *MLH1* promoter and for the V600E hot spot mutation on *BRAF*. Methylation of the *MLH1* promoter is an epigenetic event that results in an MSI-H phenotype [15]. The V600E hot spot mutation on *BRAF*, a gene involved in the RAS/RAF protein kinase pathway [16], is more frequently detected in MSI-H than in stable tumours and is mutually exclusive with germline MMR deficiency [17, 18]. The methylation of the promoter region of *MLH1* was determined by methylation-specific PCR and after sodium bisulfite modification of DNA as reported previously [18]. Mutational analysis of the *BRAF*-V600E hotspot was performed by automatic sequencing. The fragment encompassing exon 15 was amplified by PCR in all carcinoma

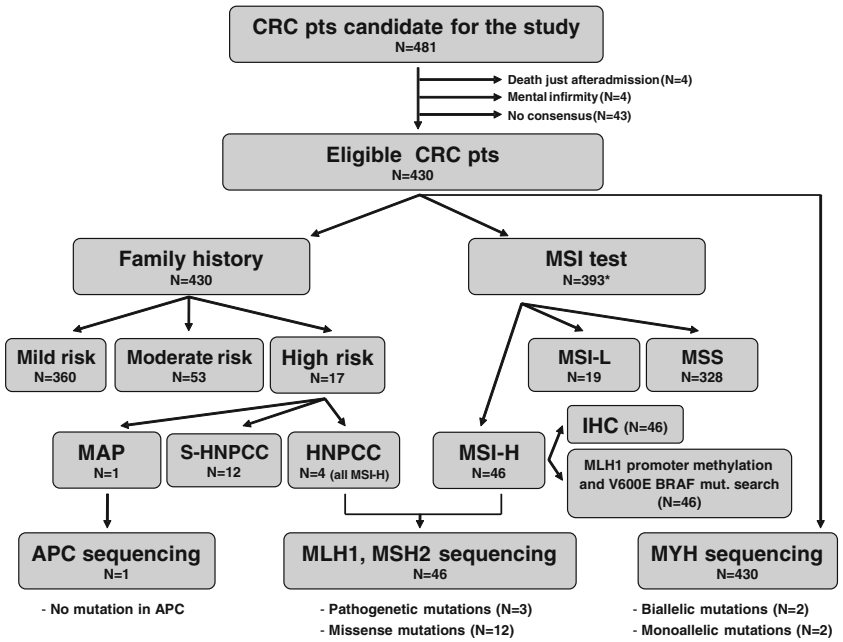

Fig. 1 Analytic strategy of the perspective part of the study and brief summary of the results. *Asterisk* 20 patients refused the consensus to store their biological samples and in 17 cases the MSI test failed for technical reasons. *CRC* colorectal cancer, *MSI* microsatellite instability, *IHC* immunohistochemistry, *AFAP* attenuated familial adenomatous polyposis, *HNPCC* hereditary non-polyposis colorectal cancer syndrome, *s-HNPCC* suspected HNPCC

samples. Primer sequences and PCR conditions were based on those reported previously [19].

Immunohistochemical staining

Immunohistochemistry for MLH1, MSH2, MSH6 and PMS2 proteins was carried out in all MSI-H tumours. Slides 7 through 9 were selected for immunohistochemical staining with antibodies to MLH1 (Pharmingen), hMSH2 (Oncogene Research Products), hMSH6 (Transduction Laboratories) and hPMS2 (Pharmingen) as previously described [20].

The staining pattern for each antibody was nuclear. A negative staining reaction in tumour cells was only regarded as a loss of protein expression in the presence of a positive staining reaction in stromal cells, lymphocytes and normal tissue adjacent to the cancer. The CRCs were scored as either negative (i.e. absence of detectable nuclear staining of cancer cells) or positive for MLH1, MSH2, MSH6 and PMS2 staining.

MLH1, MSH2, MSH6 and PMS2 genes mutation analysis

The screen for germline mutations on *MLH1* and *MSH2* was performed in patients whose family histories fulfilled the Amsterdam II criteria and in those with MSI-H tumours. *MLH1* and *MSH2* mutation analyses were carried out using a bidirectional sequencing on an automatic ABI3130 DNA analyzer (Applera).

MSH6 and *PMS2* mutation analyses were carried out in cases with a loss of protein expression [21]. Point mutations of a gene were searched by PCRs of genomic DNA with exon-specific primer pairs and bidirectional sequencing. Mutations were classified as pathogenetic and as variant of uncertain significance.

MUTYH and APC genes mutation analysis

All patients underwent genotyping for the most frequent mutations found on the *MUTYH* gene (*Y165C*, *G382D*, *IVS10+3A>C* and *1395-7delGGA*) [20]. Genotyping for the four mutations was performed by pyrosequencing on a PyroMark ID instrument as previously reported [21]. Mutated samples were confirmed by direct bidirectional sequencing with the di-deoxy method using different sets of primers (available upon request).

In patients with diffuse (more than ten adenomas) polyposis coli, genotyping of *APC* and *MUTYH* was performed. Moreover, genotyping of *MUTYH* was performed in all in the cases tested for the most frequent mutations that resulted in monoallelic mutations. Mutation analysis of the *APC* gene was carried out as described in previous investigations, and all exons of *MUTYH* were sequenced as previously reported [22].

Rearrangements within MLH1 and MSH2 genes

In exon, deletions and duplications in *MSH2* and *MLH1* were detected by multiplex ligation-dependent probe amplification (MLPA) [23] in DNA from patients whose tumours were MSI-H or deficient in either MLH1 (unrelated to *MLH1* methylation) or MSH2. We used kit SALSA P003 according to the protocol provided by MRC-Holland. All rearrangements identified by MLPA were confirmed in other affected relatives by MLPA and, when possible, cDNA analyses.

Results

Retrospective study

A total of 569 clinical records of CRC patients were reviewed in the 2-year period (2000–2001). Family history was inadequate in 227 cases (40%). In the remaining 342 cases, only one diagnosis of suspected HNPCC could be made.

During the study period, less than 10% of patients living in the Padua district underwent surgery for CRC outside of the Central Padova Hospital. Based on these findings, we assume that resident patients enrolled in the prospective part of the study represent at least the 90% of all CRC cases diagnosed in the Padua district and may be considered as a population-based series.

Prospective study

After excluding 51 patients because their family histories were unavailable (death in the perioperative period, $n=4$; mental infirmity, $n=4$; refusal to participate to the study, $n=43$), the remaining 430 patients were eligible for the study: 261 were residents in the Padua area and 169 came from other countries, mostly of the northern Italy. Characteristics of patients and tumours are summarised in Table 1.

Family history

Family history and clinical features were available in all patients with the following risk distribution: high risk, $n=17$ (4%) (HNPCC (fulfilling Amsterdam 2 criteria), $n=4$; s-HNPCC, $n=12$; MAP, $n=1$); moderate risk, $n=53$ (12%) and mild risk or sporadic, $n=360$ (84%). No FAP and no AFAP syndrome were observed (Fig. 1).

Table 1 Characteristics of patients and tumours

Patients, *n*	430
Male/female, *n*	260/170
Age: median (range)	67 (32–92)
Residents in Padua area, *n*	261
Tumour locations, *n*	
Proximal	139
Distal	197
Rectum	92
Multiple	2
pTNM stage	
0[a]	20
1	113
2	115
3	114
4	68

[a]Eleven cases of complete pathologic response after preoperative radiochemotherapy for rectal cancer, two cases had no residual tumour after endoscopic polypectomies of high risk T1 adenocarcinoma and seven cases of in situ adenocarcinoma

In the 261 patients living in the Padua district, neither HNPCC nor FAP or AFAP syndromes were observed. s-HNPCC, moderate risk and sporadic cases were found in 8 (3%), 32 (12%) and 221 (85%) cases, respectively.

Microsatellite instability analysis

Out of the 430 patients of the study group, 20 refused the consensus to store their biological samples and in 17 cases the MSI test failed for technical reasons (e.g. inappropriate storage of the specimens).

Of the remaining 393 cases, 46 (12%) showed MSI-H, 19 (5%) MSI-L and 328 (83%) MSS. The corresponding figures for the 233 evaluable patients resident in the Padua district were 26 (11%), 12 (5%) and 195 (84%). A MSI-H status was found in all 4 patients with clinical diagnosis of HNPCC, in 3 of 12 patients with s-HNPCC, in 5 of 53 cases at moderate risk and in 34 of 360 sporadic cases.

Immunohistochemical staining

Immunohistochemistry was performed on all 46 MSI-H tumours. In four cases, IHC was not evaluated for technical reasons. In the remaining 42 cases, 30 tumour specimens (70%) had negative IHC for MMR proteins: 21 had negative staining for MLH1, 3 for both MSH2 and MSH6 and 6 for MSH6 only. No patient showed the absence of PMS2 protein expression. Out of 21 patients with negative IHC for MLH1, 3 had germline mutations on *MLH1* gene, 14 showed *MLH1* promoter methylation and 4 showed neither mutations nor *MLH1* promoter methylation. Out of three patients that had MSH2-negative IHC, two had pathogenetic mutations in *MSH2*. Out of six patients that had MSH6-negative IHC, one had missense mutations in *MSH6* gene.

Methylation of the promoter region of the MLH1 and mutations in BRAF

All the 46 cases with MSI-H tumours were analysed for the methylation of the promoter region of *MLH1* and for the hotspot *BRAF* mutation, which were found in 25 and 14 cases, respectively (in seven cases, both alterations coexisted). Based on these findings, the *MLH1* promoter methylation and *BRAF* mutations can explain 32 (70%) of the MSI-H cases. Of the 32 cases that showed methylation of the *MLH1* promoter or *BRAF* mutation, only one case also showed a germline missense *MLH1* mutation.

MLH1, MSH2 and MSH6 germline mutations

Mutational screening for *MLH1* and *MSH2* was carried out in all the 46 cases with MSI-H tumours. Overall, three pathogenetic mutations were found in high-risk patients: *MLH1* intron 13, G->T at 1,558+1; *MSH2* ex 13, R711X

and *MSH2* ex1-6, del di 25Kb. Only common polymorphisms were found in 26 patients living in the Padua area that had tumours MSI-H.

In one patient that had MSH6-negative IHC, a missense mutations *MSH6* p.Glu983Gln was found. As previously described by Pastrello et al., this mutation seems to have no pathogenic effect [24].

MUTYH and APC genes mutation analysis

The results of the *MUTYH* mutation study have been previously reported [21] and are briefly expanded upon here. Overall, two biallelic and two monoallelic mutations were found on *MUTYH*. None of these mutations was found in patients with strong familial clustering of CRC; however, one of them, with a biallelic *MUTYH* mutation, had clinical characteristics of AFAP (>10 and ≤100 polyps at colonoscopy).

In this patient, the APC mutational analysis has identified a silent mutation c. 1959 G>A (R652R). In the Padua area, one biallelic and one monoallelic mutation of *MUTYH* were found.

Discussion

In the present prospective study, the systematic clinical (family history and colonoscopic findings) and molecular approach used to find hereditary CRC was well accepted by the patients, as demonstrated by the high rate of compliance to participate to the study. Five cases of hereditary CRCs were found using the clinical criteria (four cases that fulfilled the Amsterdam II criteria and one MAP). In three of these cases, a pathogenetic mutation was found.

Five cases (1.3%) of hereditary CRCs were found using the molecular approach (three pathogenetic mutations on MMR genes and two biallelic MUTYH mutations). Two of these cases fulfilled the Amsterdam criteria, one of which was classified as s-HNPCC, one with clinical features of MAP and another was sporadic. Overall, using both clinical and molecular approaches, the rate of hereditary CRC was 1.6% (n=7 patients).

Following the Amsterdam II criteria, we found an HNPCC incidence of about 1% in the hospital-based series and 0% in the population-based series. These results are consistent with previous reports [25–30] in which the diagnosis of HNPCC, based on family history, varied from 0.3% to 0.9%. On this regard, it is important to emphasize that the incidence of familial CRC was higher, since s-HNPCC, meaning cases with strong family history of CRC, reached 4% and 3% in the hospital and population based series, respectively. It has to note also that we searched for the most frequent *MUTYH* mutations: this approach could underestimate a small proportion of MAP cases.

It is remarkable that, as found in the retrospective part of the study, the family history that can be found in clinical records made by clinicians without a specific interest in inherited syndromes was unreliable. In fact, 40% of family histories found in the retrospective part of the study were inadequate to stratify patients into risk groups. These findings confirm data reported by others [31], showing that a trained team is very important to the detection of inherited diseases in the general population of CRC patients.

Using the MSI phenotype as the main molecular prescreening test for HNPCC, we found three pathogenetic mutations in MMR genes. Previous findings have indicated an incidence of disease-causing mutations of 0.3–3.6% in unselected series of CRCs [7, 32–35]. It is, however, likely that our findings are underestimated because the study period was too short (15 months), the observed population was not so large (Padua district accounts for some 410,000 inhabitants) and in 9% of the cases did not undergo MSI pre-screening. In cases of high MSI tumours, only *MLH1* and *MSH2* were extensively sequenced: The genes more frequently involved in the Lynch syndrome. *MSH6* and *PMS2* gene were sequenced when its deficiency could be suspected after IHC. Moreover, it is reported that a small percentage of HNPCC-related CRCs do not show MSI [36].

The strengths of this study are that we used a prospective approach, using both structured family history and molecular tools, data were collected by a small pool of trained researchers and molecular (MSI test) and pathological findings were standardized. In the majority of the other studies on HNPCC, the cancer family history was not retrieved [7] or was obtained from tumour registries or clinical records [28, 32–34], which demonstrated to have low accuracy on this regard [31]. Other studies on HNPCC incidence performed an MSI test on only tumour specimen of patients whose family history fulfilled the Amsterdam criteria [29, 30]. In regards to the incidence of *MUTYH* deficient CRCs, the few reports available show findings comparable to ours [37, 38]. To the best of our knowledge, however, the present study is the first that accounts for both MMR and *MUTYH* deficiency in the same CRC population.

Based on these considerations, we can conclude that in our hospital-based series of CRC, the incidence of HNPCC was at least 1%. The absence both of a clinical and molecular diagnosis of HNPCC in the population-based series is a bit surprising and may be explained by the small population sample observed and the short period of the study. As reported by others [39, 40], cases of s-HNPCC without evidence of *MMR* defects cannot be explained by mutations of *MUTYH* because none of the probands belonging to these families had a germline deficiency of this gene. The molecular interpretation of the so-called type X CRC families [41] is, therefore, far from being resolved.

The incidence of MSI tumours that we found (12%) is not negligible, and it is similar to that reported in other studies [7, 33, 42]. Some authors have suggested that MSI and IHC can be employed as a pre-screening test for detection of HNPCC [35]. Since we have more extensive expertise in MSI, we chose this analysis as the primary screening test, and as suggested by others [7], IHC was performed as a secondary test. Moreover, compared to IHC, it is important to note that MSI status could also have a prognostic role, and it has been suggested to be a prognostic factor in response to 5-FU-based chemotherapy [43, 44].

To further improve the selection of patients requiring MMR gene sequencing, we also used the *MLH1* promoter methylation test and the screen for the *BRAF* V600E hotspot mutation in MSI-H cases. Using this approach, we found that in 70% of MSI-H tumour cases, the sequencing of MMR genes may be spared. This finding both introduces new themes in the hypothesis of oncogenesis of sporadic CRCs [45] and helps in refining a strategy for a step-by-step screening in an unselected series of CRCs [46].

In conclusion, the family history present in hospital clinical records is not reliable to select families that are at high risk to develop CRC. In the study period, a strong family history of CRC was present in 4% of the enrolled cases. Five (1.3%) patients had clinical diagnosis of an inherited disease (four HNPCC and one MAP), and the incidence of disease causing mutation was 1.3% (three pathogenetic mutations on the mismatch repair genes and two biallelic mutations on *MUTHY*). Cases of suspected HNPCC, without evidence of MMR defects, cannot be explained by mutations of *MUTYH*. The incidence of MSI-H is approximately 12%, and interestingly, *MLH1* promoter methylation and *BRAF* hotspot mutation can explain 70% of these MSI-H cases, thus reducing the need for *MMR* gene sequencing. The interpretation of missense mutations on MMR genes and monoallelic mutations of *MUTYH* is challenging, at least in the attempt to find the genetic basis of the majority of CRC familial clustering that nowadays remains uncertain in origin.

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Conflicts of interest None

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