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Identification of predictive factors for the occurrence of predisposing *MLH1* and *MSH2* germline mutations among Sardinian patients with colorectal carcinoma

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Abstract

Factors predictive of carrying *MLH1* and *MSH2* germline mutations in patients with colorectal cancer (CRC) are as yet unknown. The aim of this population-based study, was to further define the role of *MLH1*/*MSH2* mutations through an evaluation clinic program with 362 consecutive Sardinian CRC patients. Eight *MLH1*/*MSH2* germline mutations were detected in 21 (6%) patients. Examining family cancer history, *MLH1*/*MSH2* mutations were found in 14/48 (29.2%) probands from CRC families and, among them, in 10/13 (76.9%) families fulfilling the Amsterdam criteria. The patients with low familial recurrence (two CRCs in the family) presented a much lower frequency of *MLH1*/*MSH2* mutations (2/55; 3.6%). Significantly higher rates of *MLH1*/*MSH2* mutations were found in patients with age of onset $\leqslant$45 years ($P = 0.012$) or with $\geqslant$3 affected family members ($P = 0.009$). While no significant predictive value was found for the presence of endometrial cancer within the family, earlier age of diagnosis and/or familial CRC recurrence should be considered as strong predictors for the occurrence of *MLH1*/*MSH2* mutations, and therefore useful in recommending CRC patients for genetic testing.
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1. Introduction

Colorectal carcinoma (CRC) is one of the main causes of neoplastic morbidity and mortality in all Western countries; incidence of CRC is second only to lung cancer in men and breast cancer in women [1]. In Italy, approximately 30,000 new cases of CRC are diagnosed every year, with approximately 15,000 registered deaths due to the disease [1]. CRC incidence presents little variation within the different geographical areas; in Sardinia, the standardized rate is slightly higher than that observed in the rest of Italy (76 *versus* 69 new cases per year per 100,000 inhabitants, respectively) [2].

Colorectal tumourigenesis is thought to occur by sequential accumulation of genetic mutations [3]. A multistep genetic model of tumourigenesis, based on specific genetic alterations in benign and primary malignant lesions, has been ascertained for CRC [4,5]. Genomic instability has been demonstrated to play a crucial role in the development of CRC tumours from normal

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epithelium (through onset of dysplastic lesions and adenomas, in a well-defined pathogenetic sequence) and is mostly due to defective replication fidelity [6].

Tumours with non-functional DNA mismatch repair (MMR) display genomic instability, as inferred by detection of ubiquitous somatic variation in length of microsatellite sequences [4,7]. Genetic instability has been associated with the presence of mutations in the two principal MMR genes, *MLH1* and *MSH2* (though the most prevalently alterated is *MLH1*) [8]. Patients with CRCs displaying microsatellite instability are more likely to present a family history positive for CRC, strongly suggesting a hereditary cancer predisposition [9,10]. Moreover, defective MMR mechanisms have been demonstrated in patients with hereditary non-polyposis colorectal carcinoma (HNPCC), an inherited cancer syndrome that also predisposes to endometrial cancer, which represents the most common extracolonic malignancy in HNPCC families [11].

In recent years, numerous studies have assessed the prevalence of both *MLH1* and *MSH2* germline mutations in various cohorts [9,11–13]. However, few of them have evaluated the predictors for the occurrence of predisposing mutations in such two main MMR genes through a clinic-based population. In this study, we report such an analysis on 362 consecutive clinical CRC patients who were classified as sporadic or familial cases according to their family history of recurrence of the disease. Our data are important for further defining the likelihood of identifying *MLH1* and *MSH2* mutations in CRC cases undergoing a cancer risk evaluation program, thus providing more accurate guidance to patients and their families for genetic counselling and testing. Moreover, the study assessed the real contribution of germline mutations in MMR genes to CRC predisposition in the Sardinian population.

2. Materials and methods

2.1. Patients

A total of 362 patients with histologically-proven diagnoses of CRC were recruited from clinics at the University of Sassari and Azienda Unità Sanitaria Locale 1 of Sassari (which represent the principal institutions accounting for cancer patients from the central and northern parts of Sardinia). Sardinian origin was ascertained in all cases through genealogical studies. Patients were enrolled consecutively over the past 3 years; no additional selection criteria were used for their inclusion in the study.

The family history of cancer was evaluated through specific questionnaires during the follow-up visits at the different departments of the participating institutions. Patients were classified as sporadic when no significant evidence of CRC cases in first- and second-degree relatives was registered. Low familial recurrence was defined by the presence of one additional affected family member (two CRC cases in the family). Patients were classified as familial cases when at least three family members (including the proband) were affected by CRC. Presence of the HNPCC syndrome was ascertained on the basis of the 'Amsterdam criteria': (i) three relatives with CRC, one of whom is a first-degree relative of the other two; (ii) CRC present in at least two generations; (iii) CRC diagnosed in at least one relative at age <50 years; and (iv) absence of the familial adenomatous polyposis (FAP) [9]. All information was verified through careful analysis of the hospital records; the cancer diagnosis of each affected family member was confirmed by examining the pathology report. In Table 1, patients are listed according to the total number of CRC cases, association with endometrial cancer, site of the primary tumour and age of onset. A written informed consent was obtained for both genetic counselling and blood sampling before genetic testing. The study was reviewed and approved by ethical review boards of both Institutions (University and Azienda Unità Sanitaria Locale 1 of Sassari).

2.2. Mutation analysis of the MLH1/MSH2 genes

Mutation analysis was performed on genomic DNA from peripheral blood samples of CRC patients. Genomic DNA was isolated using standard procedures. Primer sequences and amplification conditions for *MLH1*/*MSH2* mutation analysis were as previously reported [14]. Polymerase chain reaction (PCR) products corresponding to all exons and intron-exon boundaries of both MMR genes were analysed by denaturing high-performance liquid chromatography (dHPLC), as described previously [15]. All PCR products with abnormal dHPLC profiles were sequenced on the ABI3100

Table 1
Characteristics of colorectal cancer (CRC) patients

Characteristics	Patients (*n*)
Males	179
Females	183
Total	362
Family history of CRC	
Sporadic disease (1 CRC case in family)	259
Low familial recurrence (2 CRC cases in family)	55
Familial recurrence (⩾3 CRC cases in family)	48
Primary tumour site	
Right-transverse colon	69
Left colon-sigma-rectum	293
Age at diagnosis (years)	
⩽45	42
46–60	141
>60	179
Median (range)	60 (27–87)

Automated Sequencer (Applied Biosystems, Foster City, CA, USA).

In order to assess whether *MLH1*/*MSH2* germline variants detected by sequencing were mutations or polymorphisms, 103 unrelated normal individuals (corresponding to 206 chromosomes), originating from the same geographical area and with no family history of cancer, were used as controls.

2.3. Statistical analysis

Differences in dichotomous variables were evaluated by Pearson's χ^2 test. The exact coefficient for sample proportion analysis was performed to determine all significant parameters (below 0.05 level). All analyses were performed using the statistical package SPSS/7.5 for Windows.

3. Results

Among the 362 consecutively-collected clinical patients, 48 (13.3%) had a familial recurrence of CRC (presence of at least three affected members in the family). As shown in Table 1, approximately the same proportion of males and females was included in the study; median age was 60 years, range 27–87 years. The majority of patients (293/362; 80.9%) presented a localization of the primary tumour in the distal portions of the large bowels (including sigma and/or rectum) (Table 1).

Considering the 48 familial CRC patients, 24 (50%) were from families with both recurrence of CRC and presence of at least one relative affected by endometrial cancer (which represents the most recurrent extracolonic malignancy in CRC families). Thirteen (3.6%) out of 362 patients investigated for family cancer history fulfilled

Table 2
Characteristics of colorectal cancer (CRC) families

Characteristics	Families (*n*)
Type of recurrence	
Colorectal site-specific cancer families	24
Colorectal and endometrial cancer families	24
Total	48
Family members with colorectal cancer	
Three cases	39
⩾4 cases	9
Two affected generations	32
Three or more affected generations	16
Families fulfilling Amsterdam criteria	
No	35
Yes	13
Age at diagnosis of the family probands (years)	
⩽40	7
41–50	11
51–60	15
>60	15
Median (range)	56 (30–87)

Table 3
MLH1 and *MSH2* gene variants identified by mutation screening of 48 familial and 314 sporadic colorectal cancer (CRC) patients

Positive sporadic cases (%)	Positive familial cases (%)	Gene	Exon	Nucleotide	Codon	Base change	Amino acid (AA) change	Mutation effect	Mutation designation
–	1 (2.08)	*MLH1*	11	971_979	324	del9	3AA deletion	Small deletion, shortened protein	971del9
–	1 (2.08)	*MLH1*	16	1851	618	AA > CG	LYS to ARG	Missense	Lys618Arg
–	3 (6.25)	*MSH2*	1	34_35	12	insG	Stop 80	Frameshift, truncated protein	34insG
3 (0.96)	2 (4.17)	*MSH2*	1	38	13	G > T	SER to ILE	Missense	Ser13Ile
4 (1.27)	2 (4.17)	*MSH2*	6	965	322	G > A	GLY to ASP	Missense	Gly322Asp
–	3 (6.25)	*MSH2*	6	1024	342	G > A	VAL to ILE	Missense	Val342Ile
–	1 (2.08)	*MSH2*	6	1076	–	G > T	–	Splicing site alteration	IVS6+1G>T
–	2 (4.17)	*MSH2*	12	1781_1782	594	insCT	Stop 599	Frameshift, truncated protein	1781insCT

the Amsterdam criteria, indicating the presence of the HNPCC syndrome (Table 2). The majority (32/48; 66.7%) of CRC families presented two affected generations; median age of onset among family probands was 56 years (range 30–87 years) (Table 2).

Genomic DNA from cases with familial recurrence of CRC was analysed for germline mutations in *MLH1* and *MSH2* genes. All coding regions and splice boundaries of these two genes were screened by dHPLC analysis; PCR products with abnormal denaturing profiles in comparison to the normal controls were sequenced using an automated approach.

As shown in Table 3, two *MLH1* and six *MSH2* germline mutations were detected in familial cases from our series (including both the 55 patients with low familial CRC recurrence and the 48 CRC families). One family proband presented two *MSH2* germline mutations: Val342Ile and IVS6+1G>T. All eight sequence variants were absent in normal genomic DNA from 103 unrelated healthy individuals (corresponding to 206 control chromosomes); four of them seemed to be disease-causing mutations due to their predicted effects on protein function whereas the remaining missense variants should be classified as mutations with an unknown functional significance due to the lack of information about the effects on gene products. Altogether, 14/48 (29.2%) families were found positive for germline mutations in these two MMR genes. Most (12; 85.7%) of these mutations were found in *MSH2*, and only a small fraction (2; 14.3%) were in *MLH1* (Table 3). Moreover, detection of two recurrent *MSH2* mutations (Ser13Ile and Gly322Asp) among sporadic patients originating from different villages in north Sardinia seems to suggest the presence of founder effects for alterations in this gene within such a geographical area. Overall, *MSH2* mutations were found in 19/21 (90.5%) mutated CRC cases (ratio of *MSH2* to *MLH1*, approximately 9:1).

The proportion of CRC patients carrying *MLH1*/*MSH2* germline mutations was strikingly different when we considered the family history of cancer. Fourteen (29.2%) out of 48 probands from families with at least three cases of CRC had either a *MLH1* or *MSH2* mutation (Table 4). Taking into account the Amsterdam criteria, a remarkably higher prevalence of *MLH1*/*MSH2* mutations was found among the HNPCC cases tested (10/13; 76.9%) (Table 4). The patients with low familial recurrence (two CRC cases within the family) presented a much lower frequency of *MLH1*/*MSH2* mutations (2/55; 3.6%) (Table 4). To evaluate the prevalence of *MLH1*/*MSH2* germline mutations in the CRC population from Sardinia, we screened the remaining 259 sporadic patients for both the two *MLH1* and the six *MSH2* mutations identified in familial cases from our series. The *MLH1*/*MSH2* germline mutations were observed in five (1.9%) sporadic CRC cases.

Statistical analysis was also performed in order to assess the presence of any significant association between mutations in *MLH1*, *MSH2*, or either gene and phenotypic characteristics (location of the primary tumour and age of onset in patients; total number of CRC cases, presence of endometrial cancer, and number of affected generations in families). As shown in Table 4, age at

Table 4
Frequency of *MLH1*/*MSH2* germline mutations in (a) patients and (b) families with colorectal carcinoma (CRC)

(a) Subgroups (patients: *n*)	Cases positive to *MLH1*/*MSH2* mutations		*P*-value
	n	%	
Patients with familial recurrence of CRC (103)	16	15.5	
Two CRC cases in family (55)	2	3.6	
At least three CRC cases in family (48)	14	29.2	0.0092
Cases from families fulfilling Amsterdam criteria (13)	*10*	*76.9*	
Age at diagnosis (years)			
⩽45 (11)	6	54.5	0.0126
46–60 (39)	5	12.8	
>60 (53)	5	9.4	
Primary tumour site			
Right-transverse colon (18)	3	16.7	0.9011
Left colon-sigma-rectum (85)	13	15.3	
(b) Subgroups (families: *n*)	Families positive to *MLH1*/*MSH2* mutations		*P*-value
	n	%	
Three affected family members (39)	7	17.9	
⩾4 affected family members (9)	7	77.8	0.0189
Two affected generations (32)	5	15.6	
Three or more affected generations (16)	9	56.2	0.0876
⩾1 relative with endometrial cancer (24)	4	16.7	
No case with endometrial cancer in family (24)	10	41.7	0.0774

P: χ^2 test; two-tailed; 95% confidence interval (CI).

diagnosis ⩽45 years ($P = 0.012$) and presence in the family of three or more patients with CRC ($P = 0.009$) were significantly correlated with the presence of a *MLH1*/*MSH2* mutation. Considering the CRC families only, the presence of at least four affected family members was again significantly correlated with the occurrence of a *MLH1*/*MSH2* germline mutation. No other parameter in probands (primary tumour location, $P = 0.901$) or in family (three affected generations, $P = 0.087$; association with endometrial cancer, $P = 0.077$) was statistically correlated with the *MLH1*/*MSH2* mutations (Table 4).

4. Discussion

Identification of *MLH1* and *MSH2* mutation carriers represents an important step toward prevention and early detection of CRC risk. Due to environmental and genetic factors, prevalence of *MLH1*/*MSH2* mutations is variable among different populations. A real estimation of the proportion of positive and negative tests that might be expected in a referral risk evaluation clinic is therefore fundamental to provide clinical recommendations for *MLH1*/*MSH2* genetic tests, affecting patient decisions and cost/effectiveness estimates.

Due to the availability of limited information on population-based prevalence of germline mutations in mismatch repair genes, the present study represents one of the larger efforts toward the assessment of the role and the clinical implication of *MLH1*/*MSH2* mutations in CRC. In this analysis, 29% of patients with familial recurrence of CRC had a detectable mutation in *MLH1* or *MSH2*. Probands from families fulfilling the Amsterdam criteria presented a high frequency of *MLH1*/*MSH2* germline mutations (10/13; 77%). Although comprised into the range of the worldwide incidence (1–7%), the frequency of HNPCC in this series (13/362 patients; 3.6%) is higher than that reported for other southern Italian regions (1%) or for selected areas of northern Italy (0.5%) [16]. This clearly suggests that differences in HNPCC incidence may be due to heterogeneity of the genetic background, further confirming the importance of a precise knowledge of the actual HNPCC incidence in different populations.

A large proportion of CRC families have been demonstrated to harbour germline mutations of the *MLH1*/*MSH2* genes (together accounting for 80–85% of all mutations in HNPCC families [9,10,12,13,17]). Such genetic alterations have been documented at various rates in different populations, ranging from approximately 25% [18,19] to more than 90% [20] (average, approximately 50%). Prevalence of *MLH1*/*MSH2* germline mutations in Sardinian CRC families is consistent with that reported in other Western European populations (from Spain [18], Denmark [19], France [21] or Sweden [22]).

Many authors have suggested that presence of alterations in the mismatch repair gene pathway might be a marker of a tendency for replication errors in human cancers [8,9,23]. In our series, inactivation of the *MSH2* gene through genetic mutations in its coding regions seems to play an important role in colorectal tumourigenesis and cancer susceptibility (nearly all of the putative disease-causing mutations with a predicted effect on gene function were found in *MSH2*; see Table 3). On this regard, expression of the MLH1/MSH2 proteins is being evaluated by immunohistochemistry in tumour sections from patients with familial recurrence of CRC. Preliminary data indicated that a negative nuclear immunostaining for the MLH1 and MSH2 proteins was found in the patient carrying the *MLH1*-Lys618Arg germline mutation and in all five cases presenting the two *MSH2* germline mutations with a predicted effect on protein truncation (*MSH2*-34insG and *MSH2*-1781insCT), respectively. This suggested that such sequence variations indeed act as disease-causing mutations (Colombino; Cossu, Tanda *et al.*, in preparation).

Earlier age of onset was the most important predictive factor for the presence of *MLH1*/*MSH2* mutations (age at diagnosis ⩽ 45 years, $P = 0.012$). These findings are strongly consistent with the previously reported data regarding higher frequencies of defective DNA mismatch repair in younger CRC patients [4,6]. Conversely, no significant association between location of primary CRC and occurrence of *MLH1*/*MSH2* mutations was found in our series (though there is increasing evidence that proximal and distal colon tumours comprise distinct diseases with marked differences in the expression of tumour-associated proteins as well as in the level of genetic alterations [24–26]). Prevalence of *MLH1*/*MSH2* mutations in cases with familial recurrence of CRC was significantly correlated with the total number of affected family members (see Table 4).

Although referral for cancer risk evaluation may select for a reasonable percentage of mutation carriers when proband and family characteristics are well defined, these data highlight the fact that majority of patients who are evaluated at regional and national referral centers will not have detectable mutations in these two MMR genes. In previous studies, two-thirds of patients with familial CRC (originating from the same geographical area) presented a microsatellite instability; among them, germline mutations in *MLH1* or *MSH2* genes were detected in approximately 40% of such families [15], whereas the *MLH1* promoter hypermethylation was observed in an additional one-fifth of unstable CRCs [27]. Therefore, the remaining familial cases presenting neither *MLH1* nor *MSH2* inactivations (including the epigenetic modifications involved in gene silencing) may have alterations in other known MMR

genes (such as *MSH6* or *PMS2*) or genes from other pathogenetic pathways, which await to be identified.

In this study, *MSH2* mutations were approximately nine times more common that *MLH1* mutations. Although varying widely between populations, different studies have instead demonstrated a clear prevalence of *MLH1* mutations (the ratio of *MLH1* to *MSH2* mutations is approximately 2:1 worldwide) [18–22,28,29]. This variability could be due either to differences in the penetrance of *MLH1* and *MSH2* mutations or to population founder effects. A cancer-predisposing founder mutation of the *MSH2* gene has been described in HNPCC families living in widely different geographical locations in the United States of America (USA) [30]. In north Sardinia, a *BRCA2* sequence variant has already been demonstrated to be a disease-causing mutation with founder effect for breast cancer, due to the genetic homogeneity of the population from such a geographical area [31]. Although the prevalence of *MSH2* mutations found in this study in the Sardinian population was higher than expected, presence of founder effects for alterations in this gene may only be speculated (two recurrent *MSH2* missense mutations with an unknown functional significance were indeed found in patients and families originating from different villages of north Sardinia).

The presence of defective or inactive MMR genes has also been demonstrated to predispose to endometrial cancer (a high incidence of this disease has been observed in HNPCC families) [6,8]. From data presented in this study, *MLH1* and *MSH2* germline mutations seem to have little or no role in predisposition to endometrial cancer (4/24 (17%) mutation-positive families presented recurrence of both colorectal and endometrial cancer in comparison to 10/24 (42%) mutation-positive families with recurrence of CRC alone; see Table 4).

As also noticed above, most of the at-risk patients from families without detectable *MLH1* or *MSH2* mutations were affected with CRC. These data support the general hypothesis that there are additional CRC susceptibility genes that remain to be identified, because the pattern of CRC in some of these families is consistent with the presence of a highly penetrant autosomal- dominant susceptibility allele. Undetected *MLH1* and *MSH2* mutations may explain some of these families; however, linkage analysis and mutation detection sensitivity estimates suggest that undetected mutations are unlikely to explain all of the families without mutations. Large genomic deletions, a known source of genetic mutations, do escape detection by both dHPLC analysis and direct sequencing. However, genomic rearrangements seem to account for only 10–20% of all *MSH2* mutations, and a lower proportion of all *MLH1* mutations, among HNPCC families [32].

This study strongly reaffirms several previous predictors (earlier diagnosis age and presence in the family of three or more CRC cases) for the occurrence of predisposing *MLH1* and *MSH2* mutations. It also provides a combined analysis of both genes in a single cohort as well as information that may be useful in defining the prevalence, mutational spectrum and penetrance of *MLH1*/*MSH2* cancer susceptibility genes. All of this information should therefore be taken into account when counselling patients about genetic testing and, in particular, when considering management strategies for patients with documented mutations.

Conflict of interest statement

None declared.

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