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Value of pedigree/clinical data, immunohistochemistry and microsatellite instability analyses in reducing the cost of determining *hMLH1* and *hMSH2* gene mutations in patients with colorectal cancer☆

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Abstract

The aim of this study was to evaluate the significance of pedigree/clinical data, immunohistochemistry (IHC) and microsatellite instability (MI) analyses in the reduction of costs of constitutional *hMLH1* and *hMSH2* gene mutation diagnosis in patients with colorectal cancers (CRC). Pedigree/clinical data were evaluated on a series of 168 patients with CRC, including 43 consecutive sporadic late-onset and 25 consecutive, definitive or suspected hereditary non-polyposis colorectal cancer (HNPCC) cases, examined by IHC and MI analyses. In the latter group, 6/25 (24%) constitutional mutations were found. We detected no germline mutations in the sporadic late-onset patients. The lowest costs (880 Euro/mutation detected) were achieved by performing pedigree/clinical data (for exclusion of late-onset sporadic CRC) in conjuction with IHC only. In this model 1/6 (17%) mutations was missed. Additional preselection by IHC and MI analyses before sequencing was required to detect all mutations. In this approach, which seems to be the most effective in the search for *hMLH1* and *hMSH2* gene mutation, the cost was 1767 euro/mutation detected. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *hMLH1*; *hMSH2*; Mutation detection; Clinical criteria; Immunohistochemistry; Microsatellite instability

1. Introduction

Hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) is an autosomal dominant disease comprising 0.5–10% of all colorectal cancers (CRCs) [1, 2]. The Amsterdam criteria used in the diagnosis of the syndrome are restrictive, identifying only a small proportion of patients being actually affected by this disease [3]. Unequivocal diagnosis of HNPCC can be established by finding constitutional mutations in one of the genes involved in the development of this disease such as *hMLH1* [4], *hMSH2* [5], *hPMS1*, *hPMS2* [6], *hMSH6* [7] and *TGFβ type II* [8]. Indeed, *hMLH1* and *hMSH2* show abnormalities in more than 90% of HNPCC families with identified germline mutations [9]. The most sensitive technique to detect such mutations is DNA sequencing. However, this procedure is expensive and is not recommended for the examination of individuals with a very low probability of carrying a mutation. In the routine diagnosis of Lynch syndrome it is thus essential to develop various methods of preselection before sequencing, thus allowing a reduction of costs without a significant loss of sensitivity in the identification of alterations within *hMLH1* and *hMSH2* genes. Over the past few years, three such methods have

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been proposed: evaluation of clinical and pedigree data, microsatellite instability (MI, RER phenotype) analysis and examination of protein expression by immunohistochemistry (IHC). An assessment of clinical findings has been recommended as a basic preselection method in order to identify groups of patients with a very high or a low probability of having germline mutations [10]. MI (RER phenotype) caused by the expansion or the contraction of short repeated DNA sequences is a characteristic feature of HNPCC [11, 12]. However, MI analyses are expensive (39 to 73% of the cost of sequencing of one gene) and technically demanding, if they have to be performed on paraffin-embedded tissues. Recently, alterations of *hMLH1* and *hMSH2* gene expression, detected by the use of IHC, have been described to be associated with the occurrence of MI [13, 14]. IHC is inexpensive (approximately 14 to 28% of the cost of evaluation for MI) and can easily be performed on paraffin blocks. It can, therefore, be expected that this technique may replace MI analyses in a significant proportion of cases. However, to date the diagnostic value of IHC analyses in the search for *hMLH1* and *hMSH2* mutations in CRC patients has been described in only a limited number of cases. Moreover, existing logistic models searching for *hMLH1* and *hMSH2* constitutional mutations do not include IHC. In order to establish an economically justified model of mutation detection in consecutive patients with CRC, it is necessary to evaluate the significance of pedigree/clinical data, IHC and MI analyses in reducing the costs of finding constitutional *hMLH1* and *hMSH2* mutations in unselected patients with CRC. Studies have to be performed on large groups of such cases with simultaneous examination of their pedigree and clinical data, IHC and MI status. No similar assessments have been published to date.

2. Patients and methods

2.1. Patients

Pedigree and clinical data were analysed in a series of 168 consecutive patients with CRC. After exclusion of familial adenomatous polyposis two groups of cases (A and B) were selected for further studies: (A) Late-onset sporadic CRC-43 persons chosen from a group of 143 consecutive patients with late-sporadic CRC diagnosed at age >40 years of age and: (a) with negative family history for CRC and cancers of the endometrium, small bowel or urinary tract; (b) without occurrence of syn- or metachronous cancers; and (B) Definitive or suspected HNPCC cases—all 25 patients without fulfilment of our criteria for group A. Pedigree and clinical data are

Table 1
Pedigree/clinical data and results of molecular analyses in cases defined as definitive or suspected of HNPCC

Case	Pedigree/clinical data	hMLH1 expression	hMSH2 expression	MI status[a]	Mutation
1	extracol, a41	Negative	Normal	3\5	*hMLH1*, exon 4, insAA, stop codon
2	sibl, a23	Normal	Normal	3\5	*hMLH1* exon 4 C-T, missense
3	vert, sibl, a44	Normal	Negative	3\5	*hMSH2* exon 4 G-T, stop
4	vert, a51	Normal	Negative	3\5	*hMSH2* exon 7 C-T, stop
5	vert, a62	Pr. negative	Normal	4\5	*hMLH1* exon 19 A-G, missense
6	vert, metachr, a34, 45	Negative	Normal	Negative	*hMLH1* exon 18 G-A, missense
7	vert, sibl, a42	Negative	Normal	3\10	Negative
8	metachr, a39, 40	Pr. negative	Normal	2\5	Negative
9	a37	Normal	Normal	3\10	Negative
10	vert, synchr, a50	Normal	Normal	3\10	Negative
11	synchr, a81	Normal	Normal	Negative	Negative
12	a40	Normal	Normal	Negative	Negative
13	a30	Normal	Normal	Negative	Negative
14	synchr, a73	Normal	Normal	Negative	Negative
15	extracol, a43	Normal	Normal	Negative	Negative
16	synchr, a51	Normal	Normal	Negative	Negative
17	a34	Normal	Normal	Negative	Negative
18	synchr, a61	Normal	Normal	Negative	Negative
19	sibl, metachr, a41, 46	Normal	Normal	Negative	Negative
20	sibl, a65	Normal	Normal	Negative	Negative
21	vert, a46	Normal	Normal	Negative	Negative
22	vert, sibl, a54	Normal	Normal	Negative	Negative
23	vert, a59	Normal	Normal	Negative	Negative
24	vert, sibl, a52	Normal	Normal	Negative	Negative
25	vert, a44	Normal	Normal	Negative	Negative

a41, CRC diagnosed at age 41 years (proband); extracol, extracolonic cancer (small bowel); sibl, siblings affected; vert, vertical transmission; metachr, metachronous CRC; synchr, synchronous CRC; pr.negative, probably negative; ins, insertion.

[a] Number of markers showing instability/total number tested.

summarised in Table 1. In cases 3, 7 and 22 the Amsterdam criteria were fulfilled and HNPCC was diagnosed definitively.

2.2. Immunohistochemistry (IHC)

IHC was performed in all cases from groups A and B, as previously described [14]. Normal colonic crypt epithelium taken from tissue adjacent to the tumour served as an internal positive control. In almost all cases, staining was observed in only some areas of the tumour or control sections. We diagnosed the unequivocal absence of protein expression in cases with negative nuclear staining in tumour tissue and occurrence of staining (medium or strong intensity) in normal epithelium. Tumours with an absence of staining in neoplastic cells and with very light nuclear staining of normal tissue were regarded as cases with probable absent protein expression.

2.3. Microsatellite instability (MI)

MI was examined in all cases from groups A and B. Normal and tumour DNA were extracted from tissues frozen in liquid nitrogen or from formalin-fixed and paraffin-embedded samples. We used 10 fluorescently tagged markers divided into two panels (I and II) : I: *BAT26*, *BAT40*, *D2S123*, *D18S58*, *D17S796*; II: *D18S35*, *D3S1611*, *D2S143*, *DCC*, *D5S346*.

PCR amplifications were performed as previously described [15–19] except the annealing temperature was 65°C for *D17S796*, 64°C for *D5S346* and 50°C for *D2S143*. Products were analysed by 672 GeneScan Software (Perkin Elmer, Foster City, CA, USA). The presence of MI was diagnosed when alterations were found in at least two of the markers from panel I, or in at least 30% of all 10 microsatellites. All tumours were evaluated by the use of microsatellites from panel I. Cases showing alterations with only one marker from panel I underwent additional examination by the use of panel II.

2.4. Sequencing

Sequencing was performed in all cases from group B and in those cases from group A which showed alterations recorded by IHC or MI analyses. All exons of *hMLH1* and *hMSH2* genes were sequenced using fluorescently labelled dideoxy chain terminators [20] from Perkin Elmer in a Model 373 automated DNA sequencer.

2.5. Evaluation of costs

The economic value of the different diagnostic models, with the use of pedigree/clinical analyses and/or IHC and/or MI examinations prior to sequencing, was measured by the number of analyses and the costs necessary to examine 1 patient and to detect one germline mutation. Admitted costs of analyses: IHC approximately 50 euro (two proteins evaluated), MI approximately 175 euro (panel of 5 markers, approximately 350 euro for 10 markers) and sequencing approximately 450 euro (one gene examined).

3. Results

3.1. Findings in group A: late-onset sporadic CRC

Normal protein expression patterns for hMLH1 and hMSH2 were detected in tumour samples from 42 of 43 patients (98%). In one case (2%) staining for hMLH1 was probably negative. MI was present in an additional 4/43 tumours (9%). *hMLH1* or *hMSH2* germline mutations were not found by sequencing in any of the cases showing alterations recorded by IHC or MI analyses.

3.2. Findings in group B: definitive or suspected HNPCC cases

Normal protein expression patterns for hMLH1 and hMSH2 were present in 18/25 (72%) tumours. In 7/25 (28%) cases abnormal expression was detected. Staining was definitively negative for hMLH1 in 3 cases (cases 1, 6, 7) and for hMSH2 in two tumours (cases 3, 4). In 2 patients (cases 5, 8) hMLH1 protein expression was probably absent (Table 1). MI was present in 9/25 cancers (36%) (cases 1–5, 7–10). *hMLH1* mutations were identified by sequencing in 4 patients (16%) (cases 1, 2, 5, 6) and *hMSH2* in 2 patients (8%) (cases 3, 4).

3.3. Economic effectiveness of different diagnostic models

The highest cost reduction was achieved when preselection by pedigree data analysis was extended by IHC. When pedigree/clinical analyses (PCD) were supported only by IHC or MI, not all germline mutations were identified—the sensitivity of detecting constitutional mutations decreased by approximately 17% (detection of 5/6 mutations (83%)). All six mutations were detected in approaches based on PCD alone, or PCD followed by IHC and MI analyses. In the latter scheme, MI analysis was limited to cases with normal protein expression and sequencing was performed only in cases showing abnormalities in IHC or MI analyses (Table 2).

4. Discussion

Our studies based on a series of 168 consecutive CRC suggest that the search for *hMLH1* and *hMSH2* constitutional mutations by sequencing is not justified in

Table 2
Number of molecular analyses and their costs in searching for constitutional *hMLH1* and *hMSH2* mutations in patients with CRC, depending on diagnostic model

Model of preselection	No. of sequenced genes	No. of MI analyses	No. of IHC analyses	No. of detected mutations (%)	Total laboratory costs for 1 patient (Euro)	Cost of one mutation detected (Euro)
PCD	50	–	–	6 (100)	900	3750
PCD + MI	18	25 + 3 25-panel I 3-panel II	–	5 (83)	520	2600
PCD + IHC	7	–	25	5 (83)	176	880
PCD + IHC + MI	13	18 + 2 18-panel I 2-panel II	25	6 (100)	424	1767

PCD, pedigree/clinical data; MI, microsatellite instability; IHC, immunohistochemistry.

patients with late-onset sporadic tumours; and in cases regarded on the basis of pedigree and clinical data as definitive or suspected of HNPCC, sequencing should be preceded by IHC extended by MI analyses only in cases without alterations at protein level.

Such a diagnostic model is justified economically. The probability of finding constitutional mutations in a group of late-onset sporadic CRC is very low and thus, by exclusion of these patients, it is possible to avoid costs related to molecular analyses in approximately 85% of cases. Application of IHC extended by MI analyses in a group of patients with non late-onset CRC allows approximately 2-fold reduction of costs of one mutation detection (3750 compared with 1767) without an observed loss of sensitivity of finding constitutional alterations.

Exclusion of late-onset sporadic CRC cases from the search for mutations has been suggested previously by Aaltonen and associates [21]. The group studied 509 consecutive CRCs in Finland and detected only one constitutional mutation among sporadic cases—in a patient with CRC diagnosed at the age of 35 years. Other authors identified mutations in sporadic CRCs only in patients <40 years of age [22–24], the only exception being in a patient with a sporadic tumour diagnosed at the age of 41 years with a germline alteration (missense or mutation or polymophism) [12]. Therefore, we decided to refer to patients older than 40 years of age as 'late-onset', with a very low probability of carrying *hMLH1* or *hMSH2* germline mutations.

The frequency of low penetrance and/or *de novo* mutations, which could lead to CRC with no previous family history of cancer, is not known in other populations. In a group of patients with late-onset sporadic CRC, we identified one tumour (2%) with a probable absence of hMLH1 protein expression and an additional 4 cases (9%) with MI. No germline mutation was detected in these patients, most likely because of the limited sensitivity of techniques in detecting mutations or hypermethylation of the *hMLH1* promoter [28]. These results indicate that in Poland, in agreement with reports from Finland, there is low frequency of germline mutations in patients from this group.

Data from the literature and our results strongly indicate that it may be of crucial significance for routine diagnostics to investigate further pedigree and clinical data in correlation with molecular findings. It seems, that some pedigree and clinical features may also constitute unambiguous indications or contraindications for efficient mutation search. Preliminary data from different centres suggests, that due to the low frequency of *hMLH1* and *hMSH2* mutations, it is probably not economically justified to screen at the molecular level patients affected by truly sporadic CRC, even if they are diagnosed under the age of 40 years. A model for the molecular diagnosis of *hMLH1* and *hMSH2* mutations, in a subgroup of patients with CRC and fulfilling at least two of the Amsterdam criteria, has been proposed by Wijnen and colleagues [25]. However, it is not applicable to other cases suspected of HNPCC. Additionally, they did not analyse the usefulness of IHC. The first efforts of defining pedigree and clinical criteria, helpful in identification of *hMLH1* and *hMSH2* mutations in a large spectrum of cases suspected of HNPCC, have been summarised by Park and colleagues [26]. Our results suggest that these criteria have to be developed further because they were matched for only 3 (cases 1, 2, 6) of 5 patients (cases 1–6) with identified germline mutations and qualified by us to a group of persons suspected of HNPCC.

The highest reduction of costs in the detection of one mutation is achieved when diagnostic analyses preceding sequencing are limited to the evaluation of pedigree/clinical data and IHC. In this scheme there was an approximately 5-fold decrease of costs of molecular analyses compared with PCD alone (Table 2). Unfortunately, in such a diagnostic model some mutations can be missed. In our series of patients it would be 1 out of 6 mutations (17%). IHC can not achieve 100% sensitivity in the detection of mutations because of the occurrence

of missense mutations—they constitute approximately 30% of all *hMLH1* and approximately 10% of all *hMSH2* germline alterations in HNPCC families [27]. Missense mutations also do not always cause abnormalities in protein expression which is measured by IHC. In our study, the absence of hMLH1 staining was recorded in 2/3 tumours from patients with *hMLH1* constitutional missense mutations.

Another limitation of IHC is technical problems related to weak staining of currently available antibodies against hMLH1 and the inappropriate quality of sections embedded in paraffin blocks. In approximately 20% of cases we had to repeat IHC analyses two or even three times.

Evaluation of MI should also be considered in a diagnostic scheme aimed at reducing the number of sequenced genes without significant loss of sensitivity. Standards to examine MI are still being improved and since our experiments were started a few years ago, not all of the microsatellites used in this study belong to the group of markers recommended by the National Cancer Institute (NCI). However, it can be expected that the results of our study would not differ significantly, even if NCI markers were used, since only one marker from panel I was different from those proposed by Boland and colleagues [29].

The main disadvantage of MI analysis is its relatively high cost. An important improvement in its application, for the purposes of routine diagnostics, was finding that five microsatellite loci should be analysed in the first run and five additional markers should be added in cases with alterations present at only one locus [30]. However, even following such an approach, we found MI analyses were approximately 3-fold more expensive than IHC with no increase in sensitivity (Table 2). An important advantage of MI analyses is their ability to identify cases with a high probability of having constitutional alterations, which do not show abnormalities at the protein level. In our study case 2 (Table 1) with *hMLH1* mutation would have been missed if preselection was limited to IHC. It argues for supporting IHC by MI analysis.

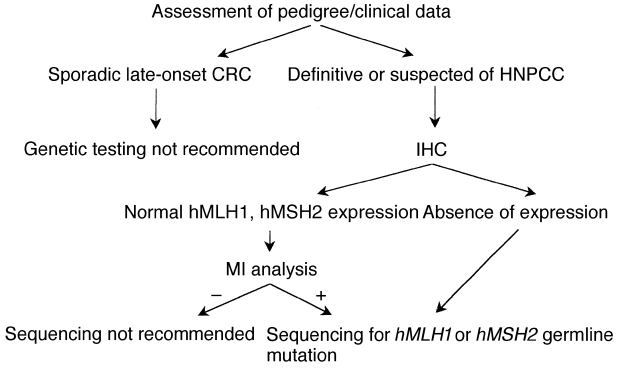

Fig. 1. Proposed diagnostic model to search for *hMLH1* and *hMSH2* germline mutations.

In summary, resources in routine mutation screening are limited. The results of our study indicate the model shown in Fig. 1 is the most effective in the identification of *hMLH1* and *hMSH2* germline mutation in patients with CRC.

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