MLH1 PROMOTER HYPERMETHYLATION OFFERS BETTER DIAGNOSTIC YIELD THAN *BRAF* V600E MUTATION IN THE ANALYTICAL ALGORITHM OF LYNCH SYNDROME

Running Head: *MLH1* HYPERMETHYLATION OFFERS BETTER YIELD THAN *BRAF* MUTATION

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Nonstandard abbreviations: MSI, microsatellite instability; IHC, immunohistochemistry; MMR, mismatch Repair; CRC, colorectal cancer; SNuPe, single nucleotide primer extension; MS-MLPA, Methylation-specific multiplex ligation-dependent probe amplification; LS, Lynch Syndrome; MSS, microsatellite stability; PBL, peripheral blood lymphocyte; WGA, Whole genome amplification; MS-MCA, methylation-specific melting curve analysis; LOH, Loss of heterozygosity, TP, True positive; FP, False positive; TN, true negative; FN, False negative; PPV, positive predictive value; NPV, negative predictive value

Human Genes: *BRAF*, v-raf murine sarcoma viral oncogene homolog B1; *MLH1*, human mutL (E. coli) homolog 1; *MSH2*, human mutS (E. coli) homolog 2; *MSH6*, human mutS (E. coli) homolog 6; *PMS2*, human PMS2 postmeiotic segregation increased 2 (S. cerevisiae)

ABSTRACT

Background:

The analytical algorithm of Lynch syndrome is increasingly complex. The sensitivity of MSI status and /or IHC status in the selection of those patients candidate to have Lynch syndrome can be improved. Two somatic alterations in colorectal tumors, *BRAF* V600E mutation and *MLH1* promoter hypermethylation, associated with the sporadic nature of MSI, have been proposed as additional prescreening methods that might improve diagnostic yield. The aim of this study was to assess the clinical usefulness of both somatic alterations in the identification of germline *MMR* gene mutations in patients with a familial aggregation of colorectal cancer.

Methods: A set of 122 tumors from individuals with a family history of colorectal cancer (CRC) that showed MSI and/or loss of MMR protein expression. *MMR* germline status was assessed and mutations were detected in 57 cases (40 *MLH1* and 15 *MSH2* and 2 *MSH6*). *BRAF* V600E mutation was assessed by Single Nucleotide Primer Extension (SNuPE). Hypermethylation status of regions C and D of *MLH1* promoter was assessed by Methylation Specific-MLPA in a subset of 71 cases with loss of MLH1 protein. Methylation-specific melting curve analysis (MS-MCA) and pyrosequencing were also used. A cost-effectiveness analysis was performed.

Results: *BRAF* mutation was detected in 14 of 122 (11%) cases. In 1 of 14 cases mutation was detected in the tumor of an *MLH1* mutation carrier. Sensitivity of the absence of *BRAF* mutations for depiction of Lynch syndrome patients was 98% (56/57) and specificity was 22% (14/65). Taken into account cases with loss of MLH1 expression, sensitivity of *BRAF* mutation was 96% (23/24) and specificity 28% (13/47). Specificity of *MLH1* promoter hypermethylation for depiction of sporadic tumors was 66% (31/47) and sensitivity of 96% (23/24). *BRAF* mutation enabled to identify sporadic cases before *MMR* germinal mutation

study in 13 of 47 cases that increased to 31 when hypermethylation status was taken into account. Hypermethylation study of *MLH1* promoter is more cost-effective than *BRAF* mutation analysis.

Conclusion: In the context of clinical algorithm of Lynch syndrome, the study of somatic *MLH1* hypermethylation provides greater efficiency than the study of *BRAF* V600E mutation in the selection of patients for genetic testing.

INTRODUCTION aiming to 3500

Lynch syndrome (LS) is characterized by an autosomal dominant inheritance of early-onset colorectal cancer (CRC) and increased risk of other cancers (1, 2). It is caused by germline mutations in DNA mismatch repair (*MMR*) genes. *MLH1* or *MSH2* are the most commonly mutated *MMR* genes in LS, whereas mutations in *MSH6* or *PMS2* are significantly less common (3-5).

Heterogeneity in the mutations identified in DNA *MMR* genes and low percentage of hereditary tumors among familial aggregation make it expensive to test all patients in whom this condition is suspected. Microsatellite instability (MSI) is a hallmark of MMR-deficient cancers and is found in more than 90% of LS colorectal tumors (6-11). Immunohistochemical staining is used to determine the expression of MMR proteins in tumor tissue of candidate patients. Both strategies are generally accepted as prescreening procedures for genetic testing of *MMR* genes with similar clinical performance (12, 13). In spite of their evident clinical usefulness, their sensitivity is still low. In consequence, there is a need to better refine the LS diagnostic algorithm.

BRAF mutations, mainly located at V600E, are present in approximately 10% of CRCs, and in a higher proportion of MSI tumors. This mutation is strongly associated with the microsatellite instability phenotype due to *MLH1* inactivation that results from promoter methylation (14-20). It has been used to distinguish LS-associated tumors from sporadic MSI-positive tumors (14, 15, 17, 21-25). The lack of *BRAF* mutations identify with high sensitivity (96 – 100%) and lower specificity (22 - 100%) those cases associated with LS (14, 15, 17, 21-25) . Occasionally, *BRAF* mutations have been detected in tumors from LS patients (26).

Methylation of the *MLH1* promoter, leading to a loss of MLH1 expression, is also strongly associated with sporadic MSI-positive CRCs. *MLH1* promoter hypermethylation has been also utilized in the selection of patients with sporadic MSI-positive tumors that will not be tested for germline mutation (24, 25, 27-32). However, the identification of hypermethylation in a limited number of LS tumors has made its use controversial (14, 33-35).

For both *BRAF* mutation detection and the study of *MLH1* promoter hypermethylation it is critical to assess their analytic and clinical validity as well as its cost-effectiveness prior to routine implementation in the clinical setting. Issues that affect screening include the accuracy, sensitivity, and specificity of the test, the benefit to the patient, the possible negative ramifications of the results, and the cost (12, 36).

The aim of this study was to compare the diagnostic yield and cost-effectiveness of *BRAF* V600E mutation detection and analysis of *MLH1* promoter hypermethylation in the selection of candidate patients to *MMR* gene germline mutation analyses in a large series of cases with familial aggregation for which *MMR* gene status was studied.

SAMPLES AND PATIENTS

A total of 122 colorectal tumors with MMR deficiency (as evidenced by MSI or combined MSI and loss of MMR protein expression) were obtained from individuals with a family history of CRC attended at our Cancer Genetic Counseling Unit between 1999 and 2008. Forty-three patients met Amsterdam criteria, 48 met revised Bethesda criteria and 12 cases showed other types of CRC familial aggregation. In all cases *MMR* germline mutation status was assessed by direct sequencing and Multiplex Ligation-dependent Probe Amplification

(MLPA). Fifty-seven of the 122 MSI tumors were from LS patients (40, 15 and 2 with *MLH1*, *MSH2* and *MSH6* mutations, respectively). Clinico-pathological information was recorded. In addition, a series of 48 MSS tumors from patients showing CRC familial aggregation and 73 sporadic CRC from a case-control study (37) were also analyzed. Informed consent was obtained from all patients, and the ethics committee approved this study.

DNA extraction of colorectal tumor tissue from paraffin-embedded material was done after enrichment for tumor cells using the QIAmp DNA Mini Kit (Qiagen, CA). DNA from RKO colorectal tumor cell line (American Type Culture Collection) was used as a biallelic *MLH1* methylation control. DNA from COLO 201 colorectal cell line (kindly provided by Dr. Richie Soong) and SK-MEL-28 melanoma cell line (American Type Culture Collection) were used as controls of homozygous *BRAF* V600E mutation. To generate unmethylated DNA, PBL DNA was amplified using the REPLI-g kit (Qiagen).

MATERIALS AND METHODS

Detection of *BRAF* V600E mutation

A 196-bp region of human *BRAF* exon 15 spanning the hotspot mutation c.1799T>A (V600E) was amplified by PCR with *BRAF*_PCR primers (Supplemental Data Table 1). PCR reaction was performed in 30µl of 1.5mM $MgCl_2$, 200µM dNTPs, 20-100ng genomic DNA, 0.5µM of each primer and 1U Taq DNA Polymerase (Invitrogen, USA) for 35 cycles with annealing temperature of 55 ºC. The PCR products were purified using Illustra™ GFX™ DNA and Gel Band Purification kit (GE Healthcare, UK). Sequencing analysis of PCR products was performed with BigDye Terminator v.3.1 Kit (Applied Biosystems, USA) using amplification primers.

BRAF V600E mutation detection was performed by Single Nucleotide Primer Extension (SNuPE) using the ABI PRISM® SNaPshot® Multiplex Kit (Applied Biosystems) with the

153 *BRAF*_SNuPe primer (as described in Supplemental Data Table 1). Analytical sensitivity of
154 *BRAF* V600E mutation analysis was assessed in serial dilutions of homozygous V600E
155 mutated DNA from SK-MEL-28 and COLO 201 cell line at final percentage of 100%, 30%,
156 10%, 5%, 3%, 1%, 0.5% and 0% with wild type genomic DNA from PBL, previously
157 quantified using Quant-iT^{TM} PicoGreen dsDNA reagent (Invitrogene).
158
159 **Detection of *MLH1* promoter methylation status**
160 ***Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA)***
161 SALSA MS-MLPA ME011 kit (MRC Holland, Amsterdam) is based on the use of probes
162 that contain a digestion site (or occasionally 2 digestion sites) specific for the methylation-
163 sensitive HhaI enzyme. All reactions were carried out using 100 - 150 ng of DNA. Kit
164 includes 5 probe pairs in *MLH1* promoter (with the respective HhaI sites located at -638, -
165 402, -251/-245, -8 and +220 relative to the initiating transcription; GenBank accession
166 number U26559) that cover 5 independent regions: regions A to D of the promoter and intron
167 1. Analytical sensitivity was assessed in serial dilutions (100%, 50%, 20%, 15%, 10%, 1%
168 and 0%) of RKO DNA and unmethylated REPLI-g-amplified DNA, previously quantified
169 using Quant-iTTM PicoGreen dsDNA reagent (Invitrogene).
170 Intra and interexperiment variability was assessed using a tumor sample showing methylation
171 values close to 20% because similar value has been proposed as a meaningful cut-off value in
172 previous studies (38, 39). Ten replicates in two independent experiments were analyzed.
173
174 ***Methylation-Specific Melting Curve Analysis (MS-MCA)***
175 MS-MCA method consists in a Real Time PCR followed by temperature dissociation (40) on
176 DNA previously treated with sodium bisulfite, using the EZ DNA Methylation-Gold Kit
177 (Zymo Research, Orange, CA). The amplification primers were designed to avoid CpG

targeting. (for details see supplemental table 1). Melting curve analysis was performed by heating the PCR products from 60ºC to 98ºC with an increase of 0.2°C/s whereas fluorescence was monitored continuously. Analytical sensitivity of the method was assessed as described above.

Pyrosequencing

Two µl of bisulfite converted DNA were used in a PCR reaction of the regions of interest using HotStar Taq master mix (Qiagen) and biotin labeled primers. Primers were designed using the Pyromark Assay Design Software 2.0 (Qiagen). For experimental conditions and primer sequences see Supplemental Data Table 1, Analytical sensitivity was assessed as described above.

Analysis of *MLH1* loss of heterozygosity (LOH) in the *MLH1* mutation carrier

Allelic imbalances and copy number variation were analyzed using SNuPe technique and MLPA respectively. Experimental conditions are described in Supplemental Datal Table 1.

Cost-effectiveness analysis

A decision model was developed to estimate the number of mutation carriers and the incremental costs of alternative case-finding methods for detecting *MMR* mutation carriers among individuals with positive molecular test in tumors (MSI and/or loss of expression of MLH1). Three potential strategies were considered. Strategy 1 (*BRAF – MLH1* mutation analysis) involved *BRAF* V600E testing of all individuals with positive molecular test in tumors. If no mutation was detected, DNA testing for *MMR* mutations followed. Strategy 2 (Hypermethylation - *MLH1* mutation analysis) involved testing for *MLH1* hypermethylation

of all individuals with positive molecular test. If hypermethylation was absent, DNA testing for *MMR* mutations followed. Strategy 3 (*MLH1* mutation analysis) involved direct *MMR* mutation testing of all individuals with positive molecular test in tumors.

Pathway probabilities were attached to the decision tree (Supplemental Data Figure 1). For each strategy, the number of individuals tested, the number of mutations detected and missed, the number of false-positive results, and the number of *MMR* mutations detected in first- and second-degree relatives was computed. In addition, we attributed resource valuations to each event in order to calculate a total cost for each strategy (Supplemental Data Table 2). The health outcome was defined in terms of additional *MMR* mutations detected.

Two independent assessments were performed: one that considered costs and outcomes for proband only, and a second that included proband and their first- and second-degree relatives. Following a healthcare perspective, only direct medical costs were used for analysis. The decision model was run using Microsoft Excel (Microsoft Corp., Redmond, Washington).

Sensitivity of the cost-effectiveness assessment was performed using a one-way analysis where each parameter is tested over a range of values while holding all other parameters at their base-case values. Ranges for each variable were based on a literature review or on expert opinion whenever no range data were available.

RESULTS

Usefulness of *BRAF* V600E mutation analysis in the identification of LS tumors

SNaPshot consistently detected the *BRAF* V600E mutation when it was present in 5% of all alleles analyzed (Supplemental Data Figure 2). Assay was initially validated in a set of 73 selected sporadic CRC (37) [24 MSI and 49 MSS]. In these tumors *BRAF* mutation was identified in 5 of 24 (20%) MSI and 2 of 49 (4%) MSS tumors.

A set of 170 familial tumors were studied, 48 MSS and 122 MSI. *MMR* germline mutations were found in 57 (40 *MLH1*, 15 *MSH2* and 2 *MSH6*) of the 122 (47%) MSI tumors. *BRAF* mutations were detected in 3 of 48 (6%) MSS tumors and in 14 of 122 (11%) MSI tumors. One *BRAF* V600E mutation was identified in a tumor from a germline *MLH1* mutation carrier, and the remaining 13 were found in cases with no identified germline *MMR* mutations. Absence of *BRAF* mutations was highly sensitive (98%) for the identification of LS tumors. Specificity was 22% (Table 1). All cases harboring *BRAF* V600E mutations were associated with loss of MLH1 expression. Restricting the analysis to the 71 tumors with loss of MLH1 protein expression (24 LS and 47 non-LS tumors), the absence of the mutation showed a sensitivity of 96% (23/24) and a specificity of 28% (13/47) for depiction of LS tumors (Table 1).

Usefulness of *MLH1* promoter methylation analysis in the identification of LS tumors

MS-MLPA could consistently detect the presence of 10% methylated alleles for the 5 regions analyzed (regions A to D and intron) (Figure 1). Intra and interexperiment variability of MS-MLPA was low within the range of 1%. No differences were observed among the five probes utilized. However, for clinical purposes and following recommendations by Deng et al (41), only positive results for regions C and D were taken into account because this methylation correlates with the loss of expression of *MLH1*.

In order to assess the potential for quantitative assessment of MS-MLPA, the reconstituted samples were also analyzed using pyrosequencing, the gold-standard technique for methylation quantification. Of note, the analytical sensitivity of MS-MLPA was similar to that of pyrosequencing (Supplemental Data Figure 3). For this experiment, DNA from PBL was the unmethylated control since the fragmented bisulfite treated DNA from Repli-G

amplification did not yield consistent results. Pyrosequencing sensitivity was 5% for C-region and 10% for D-region (Supplemental Data Table 3). The intensity of the methylation signal of MS-MLPA-targeted CpGs is average compared to all CpG sequence as assessed by pyrosequencing. Of note, MS-MLPA and pyrosequencing showed similar results regarding quantitation of the signal.

In the set of 71 tumors with MLH1 loss, MS-MLPA analysis evidenced *MLH1* promoter hypermethylation in 32 cases when a cut-off value of 20% was used (Table 1). Hypermethylation was present in 1 of 24 LS tumors and 31 of 47 non-LS tumors. Absence of hypermethylation showed a sensitivity of 96% and a specificity of 66% for LS identification. Concomitant C and D regions hypermethylation was observed in 27 cases, whereas 2 cases displayed exclusive *MLH1* C-region methylation and 3 tumors displayed exclusive D-region methylation (Figure 2). Interestingly, in 41 of the 71 tumors, methylation was detected in A and/or B *MLH1* promoter regions (Figure 2). If we would have included methylation in A or B promoter regions when scoring the samples, 34 cases would have been correctly classified as sporadic tumors. However, six cases would have been misclassified as false positive cases. Finally, the combination of *BRAF* V600E and *MLH1* hypermethylation did not yield any additional value (Table 1).

In our experience, the 20% cut-off value for MS-MLPA for *MLH1* promoter assessment seems to be useful in this clinical setting. However, the use of distinct cut-off values for MS-MLPA analysis influences the prevalence of methylation and consequently its putative clinical usefulness (Table 1). Thus, we decided to explore whether alternative methods to assess methylation status could offer better yield in the clinical setting. A methylation-specific melting curve analysis (MS-MCA) test was developed to evaluate methylation in C- and D-

regions of *MLH1* promoter. In reconstituted samples MS-MCA was able to detect a methylated allele when it was present in 5% of all alleles (Supplemental Data Figure 3). Next, six tumor samples for which methylation levels were estimated between 5 and 20% by MS-MLPA were analyzed. MS-MCA scored as methylated one LS case that MS-MLPA classified as unmethylated. On the other hand, MS-MCA failed to identify as methylated the case showing 20% methylation levels by MS-MLPA (data not shown). Altogether in this small set of cases, MS-MCA does not seem to add value in those cases with borderline values according to MS-MLPA.

In all, only one LS-associated colorectal tumor harbored somatic hypermethylation of the *MLH1* promoter and a *BRAF* mutation. This tumor arose in a patient that fulfilled Bethesda criteria and was diagnosed of metachronous CRC, the first one diagnosed at 23 years old (right colon) and the second at the age 43 (sigma, pT3pN2M0), being the second tumor the one analyzed. Family history included a diagnosis of CRC of his mother and his father developed a gastric cancer. Tumor tissue study revealed *MLH1* hypermethylation in C- and D-regions and a *BRAF* V600E mutation (Supplemental Data Figure 4). Neither somatic copy number variation of the *MLH1* gene nor LOH was evidenced. While the tumor displayed the typical molecular profile associated with sporadic MSI tumors, the patient was a carrier of the founder Spanish c.1865T>A MLH1 (L622H) pathogenic mutation (42).

Cost-effectiveness analysis

The three strategies [BRAF - *MLH1* mutation analysis (Strategy 1); Hypermethylation - *MLH1* mutation analysis (Strategy 2); DNA testing of all individuals (Strategy 3)] (Supplemental Data Figure 1) were analyzed in a hypothetical cohort of 1000 newly diagnosed colorectal cancer patients with loss of MLH1 expression (Table 2). DNA testing of

all probands (Strategy 3) is anticipated to identify all expected carriers (n=338) (Table 2). Strategies 1 and 2 identified the same number of carriers (n=324), but strategy 1 (absence of *BRAF* mutation) associates with a very high number of false-positive results when compared to strategy 2 (lack of promoter hypermethylation) (479 vs 165). When first- and second-degree relatives were considered along with probands, the number of identified MMR mutation carriers increased (n=810) improving the clinical impact of the screening. Strategy 3 was able to identify 35 additional cases (Table 2).

Strategy 2 (Hypermethylation as a pre-screening test) offered the lowest cost per mutation detected (Table 2). When probands were considered, incremental costs for the identification of an additional mutation was €2,212 for strategy 2 while for strategy 3, the most specific, the incremental costs were exceedingly higher (27,220€) (Table 2). When costs and benefits were calculated including first- and second-degree relatives, the incremental cost for Strategy 2 and Strategy 3 was €846 and €7.991, respectively (Table 2).

However, these strategies are sensitive to a number of parameters. Strategy 3 is highly sensitive to the performance (sensitivity and specificity) of the *MLH1* hypermethylation testing as well as the prevalence of *MMR* mutations. On the other hand, strategy 2 is also sensitive to the specificity of the MLH1 test as well as the prevalence of *MMR* mutations but to a lower extent. Importantly, results do not vary whether first-and second-degree relatives are included or not in the analysis (Figure 3).

DISCUSSION

Two somatic molecular determinations (lack of *BRAF* mutations and absence of *MLH1* methylation) have been proposed as good screening tests for the identification of patients candidate for *MLH1* germline testing. Here, we show that *MLH1* hypermethylation analysis on tumor biopsies, as assessed by MS-MLPA, outperforms *BRAF* mutation in the selection of these patients being more cost-effective.

The association of *BRAF* mutation with the *MLH1* hypermethylation and the MSI phenotype resulted in its evaluation as a potential pre-screening tool in the LS diagnostic algorithm (14, 15, 17, 21, 23, 24, 43) (Table 3 and Supplemental Data Table 4). In agreement with previous reports, the sensitivity of the absence of *BRAF* mutation is very high in identifying *MLH1* mutation carriers (21, 22, 25, 44) (Table 3 and Supplemental Data Table 4). This association, however, is not perfect. In our series, a single false negative was identified adding to the increasing number of LS tumors harboring a *BRAF* mutation (26). In contrast, its specificity is low (less than 22% when only cases showing loss of MLH1 protein were considered). Two factors may account for this observation. On the one hand, the low prevalence of *BRAF* mutations observed in our population (11% of MSI tumors and 20% of those lacking MLH1 protein expression). This is in the lower range of reported series (21, 22, 25, 44) but likely to reflect the experience of referral centers. On the other hand, the number of LS cases and *MLH1* germline carriers included that helps in more accurate estimates.

Methylation of *MLH1* promoter was assessed in the same set of 71 tumors. Sensitivity again was very high with a single false negative that also shared a *BRAF* mutation. The lack of *MLH1* promoter hypermethylation showed a sensitivity of 66% for LS depiction. Again, this

is in the lower range (57-100%) of reported series (16, 22, 25, 44) (Table 3 and Supplemental Data Table 4)) and associates with a relatively low prevalence of hypermethylation in our population (42%). These observations support the notion that the characteristics of the population analyzed influences the performance of the technique.

A number of techniques have been proposed to study the hypermethylation in tumor tissues (27, 30, 35, 45, 46). However, no technique has shown an evident superiority in the routine clinical setting. MS-MLPA, has previously shown a better yield in the clinical diagnostic laboratory than other quantitative techniques as Methylight (25). In our hands, MS-MLPA has proved a robust option, with low variability and good analytical sensitivity when using the highly degraded DNA extracted from paraffin blocks. This good performance associates with the use of short probes that work well in the fragmented DNA and the fact that it no bisulfite conversion, with the corresponding DNA damage, is needed.

For any methylation technique, the definition of clinically meaningful cut-off values is critical. We have validated the previously defined arbitrary 20% cut-off value (38, 39) to score cases as lacking/harboring *MLH1* hypermethylation. Other studies have used a 15% threshold obtaining similar performance (25). In our hands, the use of the 15% threshold would have resulted in an increase of false positive results (3). Also, we have confirmed that considering only C- and D-regions of *MLH1* promoter, those less sensitive to constitutive methylation (41, 47), yields the best performance in the diagnostic setting (22, 25, 44). We have explored other methodological alternatives such as as MS-MCA and pyrosequencing. The quantitative assessment obtained by pyrosequencing is attractive, however its application to the study of DNA obtained from paraffin samples is not straightforward. In our opinion, MS-MLPA seems to be the methodology of choice in assessing MLH1 hypermethylation

when formalin-fixed paraffin-embedded tissues originating from different centers are to be used.

A single false negative case, in a member of a Spanish founder mutation family (L622H mutation carrier), has been identified (42). Interestingly this case shows both a *BRAF* mutation and promoter hypermethylation. Walsh et al. have reported a similar case (26): a member of LS family that also showed predisposition to develop serrated polyps in the colorectum. The occasional presence of *BRAF* mutations in this setting challenges the universality of the link of *BRAF* mutation to non-LS families (14, 15, 17, 21-25). Some evidence suggests that non-LS MSI-H cases may originate from sessile serrated adenoma (48-53). Interestingly, the patient in this paper developed 3 hyperplastic polyps (in the sigma and in anal margin) and a tubular adenoma.

The concomitant existence of *MLH1* mutation and hypermethylation of its promoter in the literature has been extensively documented (14, 33-35). However in our setting its prevalence is very low, supporting its utility. Although LOH analysis was not completely, we assume that this mechanism did not present in *MLH1* gene after comparing normal tissue with tumor using SNuPe and MLPA analyses. If we assume that *BRAF* mutation is associated to *MLH1* inactivation by hypermethylation (14, 18, 19, 54, 55) in this case it would be the second hit to would inactive *MLH1*.

MLH1 hypermethylation analysis does not only outperform *BRAF* mutation analysis but is more cost-effective, in terms of incremental cost per additional mutation carrier detected. It is also more cost-effective than direct testing for all candidate patients. This is an important deed, since direct germline mutation study is the most time-consuming and expensive step.

Our results are in line with those by Pérez-Carbonell et al (25) that using the less complex cost-minimization approach reached similar conclusions. The cost-effectiveness analysis is highly sensitive to changes in the prevalence of germline mutation in the population analyzed. In this specific population, the prevalence of *MLH1* germline mutation is 47%, which likely reflects the population assessed in referral centers. Also, these analyses are quite sensitive to the operating characteristics of *MLH1* methylation detection technique further reinforcing previously discussed issues about MS-MLPA.

The present study reflects the experience of a Cancer Genetics Unit. The inclusion of a significant number of tumors from patients with familial aggregation that were analyzed for germline mutations status provides with a solid comparison with somatic analysis. The direct comparison of two molecular analysis, *BRAF* mutation and hypermethylation is also relevant since only a handful of studies have analyzed one or both strategies using a significant number of tumors from sporadic and LS cases (15, 35) (Supplemental Data Table 4).

In conclusion, somatic hypermethylation of *MLH1* is an accurate and cost-effective pre-screening method in the selection of those cases candidate for germline analysis when LS is suspected and MLH1 protein expression is absent. When using MS-MLPA the occurrence of false negatives is low making it a reasonable option in the diagnostic algorithm of LS being aware that in some LS cases may not be identified. The present study adds significant evidence supporting the introduction of this prescreening method in the routine diagnostic setting of LS

LEGEND TO FIGURES

Figure 1. Analytical sensitivity and experimental variability of *MLH1* promoter hypermethylation analysis as assessed by MS-MLPA**.** To evaluate the performance of MS-MLPA we tested serial reconstitutions of methylated alleles in increasing amounts of unmethylated alleles ranging from 100% to 0% (100, 50, 20, 15, 10, 1, 0). A linear relationship was detected between observed methylation and predicted methylation. Intra- and inter-assay variability was evaluated analyzing a clinical sample showing methylation values close to 20%. Variability was low within the range of 1% after analyzing ten replicates in two independent experiments.

Figure 2. Detailed methylation patterns of the *MLH1* gene promoter, as assessed by MS-MLPA, of the 71 familial colorectal cancer tumors showing loss of MLH1 protein expression. The 5 regions of the CpG island targeted by the selected probes are shown. Samples harbouring or lacking germline *MLH1* gene mutations are separately described. Box highlights the methylation pattern of the informative C and D regions.

Figure 3. Most relevant parameters influencing the cost-effectiveness of the distinct strategies utilized.

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