METHODS

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Quantitative PCR High-Resolution Melting (qPCR-HRM) Curve Analysis, a New Approach to Simultaneously Screen Point Mutations and Large Rearrangements: Application to *MLH1* Germline Mutations in Lynch Syndrome

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ABSTRACT: Several techniques have been developed to screen mismatch repair (MMR) genes for deleterious mutations. Until now, two different techniques were required to screen for both point mutations and large rearrangements. For the first time, we propose a new approach, called "quantitative PCR (qPCR) high-resolution melting (HRM) curve analysis (qPCR-HRM)," which combines qPCR and HRM to obtain a rapid and cost-effective method suitable for testing a large series of samples. We designed PCR amplicons to scan the *MLH1* gene using qPCR HRM. Seventy-six patients were fully scanned in replicate, including 14 wild-type patients and 62 patients with known mutations (57 point mutations and five rearrangements). To validate the detected mutations, we used sequencing and/or hybridization on a dedicated *MLH1* array–comparative genomic hybridization (array-CGH). All point mutations and rearrangements detected by denaturing high-performance liquid chromatography (dHPLC)+multiplex ligation-dependent probe amplification (MLPA) were successfully detected by qPCR HRM. Three large rearrangements were characterized with the dedicated *MLH1* array-CGH. One variant was detected with qPCR HRM in a wild-type patient and was located within the reverse primer. One variant was not detected with qPCR HRM or with dHPLC due to its proximity to a T-stretch. With qPCR HRM, prescreening for point mutations and large rearrangements are performed in one tube and in one step with a single machine, without the need for any automated sequencer in the prescreening process. In replicate, its reagent cost, sensitivity, and specificity are comparable to those of dHPLC+MLPA techniques. However, qPCR HRM outperformed the other techniques in terms of its rapidity and amount of data provided. Hum Mutat 0, 1–9, 2009.

KEY WORDS: *MLH1*; melting curve analysis; HRM; quantitative PCR; prescreening method; MMR

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC; MIM# 114500), or Lynch syndrome, is an autosomal dominant disease with early onset of colorectal cancer and other associated tumors (endometrium, small bowel, and urinary tract) [Lynch and de la Chapelle, 1999].

Lynch syndrome is caused by deleterious mutations in the genes involved in the DNA mismatch repair system (MMR). In these genes (*MSH2*, *MLH1*, *PMS2*, and *MSH6*), the majority of causative mutations (90%) have been found in *MSH2* (MIM# 120435; GenBank: NM_000251.1) and *MLH1* (MIM# 120436; GenBank: NM_000249.2) [Kurzawski et al., 2006].

Germline mutations in *MLH1* and *MSH2* are mainly point mutations (small deletions/insertions of few nucleotides, splice-site changes, and nonsense and missense mutations). Different prescreening methods have been proposed, such as denaturing high-performance liquid chromatography (dHPLC) [Holinski-Feder et al., 2001], denaturing gradient gel electrophoresis (DGGE) [Wijnen et al., 1995], and single-strand conformational polymorphism (SSCP) [Beck et al., 1997]. dHPLC is considered the gold standard for prescreening and has been

Additional Supporting Information can be found in the online version of this article.

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described as the most sensitive method for *BRCA1* prescreening [Gerhardus et al., 2007]. Major drawbacks of the dHPLC method are chemical waste, the cost of maintenance, and the need for post-PCR manipulations. dHPLC does not allow high-throughput mutation screening, since there is only one sample per run. Development requires a long and intensive optimization process. For genes with a large number of polymorphisms, such as *MSH6*, the time taken for dHPLC runs and the need to sequence a large number of amplicons have led some laboratories to select direct DNA sequencing instead of dHPLC for all *MMR* genes [Wahlberg et al., 1999]. Even if a sequence is the endpoint in molecular analysis, the cost of sequencing a whole gene is higher than that of processes with a prescreening method [Sevilla et al., 2003].

Other types of germline mutations consist of more complex rearrangements with deletion or duplication affecting a large part of the gene. They account for up to 15% of all pathogenic mutations in *MSH2* and *MLH1*. The frequency of large rearrangements in *MSH2* compare to *MLH1* depends on the studied population [Charbonnier et al., 2002; Wang et al., 2003].

To detect large rearrangements, alternatives to sequencing include Southern blotting, real-time PCR gene dosage, the protein truncation test, and semiquantitative multiplex PCR assays. Semiquantitative multiplex PCR assays such as multiplex ligation-dependent probe amplification (MLPA) and quantitative multiplex PCR of short fluorescent fragments (QMPSF) have advantages of speed, cost, and reliability, and are routinely used in many laboratories. However, they have some limits. First, neither is integrated in point mutation scanning technologies, and both require an automatic sequencer to analyze fragments. In MLPA, overnight ligation (16 hr) is required following tube opening. The results are very sensitive to DNA quality, but few quality controls exist. Some false positives have been reported, such as false duplication. We report an isolated duplication in exons 1–2 of *BRCA2* detected by MLPA. A specific BRCA1/BRCA2 array-CGH [Rouleau et al., 2007] showed nonspecific duplications in the 5′ and 3′ regions of the both genes, reflecting a quality problem with this sample, as they were not detected by MLPA. Generally, there are no indicators available to exclude low quality samples, except in the case of aberrant results for several exons (duplication, deletion, and aberrant ratio).

For all these reasons, and to standardize the prescreening process, it is necessary to find new approaches.

Recently, high-resolution melting (HRM) curve analysis has been proposed in several publications as a routine prescreening method for cancer predisposition genes, such as *BRCA1/BRCA2* [de Juan et al., 2008; De Leeneer et al., 2008; Takano et al., 2008]. HRM involves precise monitoring of the change in fluorescence caused by the release of an intercalating DNA dye from a DNA duplex as it denatures at high temperatures. HRM technology has been introduced on quantitative PCR (qPCR) machines. We thus tested a combination of the two approaches to detect, in a single assay, both point mutations and large rearrangements. Our approach, named "qPCR-HRM" uses real-time PCR gene dosage associated with HRM curves. All DNA germline mutations in coding and splicing regions can be prescreened by analyzing the amplification and melting curves. With less than 10% of amplicons bearing mutations (deleterious mutations, unclassified variants, and polymorphisms), *MLH1* is a good candidate for validation of a prescreening method. To investigate the capacity of qPCR-HRM to detect point mutations and large rearrangements simultaneously in the *MLH1* gene, we analyzed 57 known point mutations (three variants per exon on average) in the *MLH1* gene and five large rearrangements (three deletions and two duplications). We also analyzed 14 patients with no detectable mutations in the *MLH1* gene. The majority of mutations detected here were confirmed and characterized by sequencing and/or using a dedicated *MLH1* array-CGH as described in other studies [Rouleau et al., 2007; Staaf et al., 2008].

Materials and Methods

DNA Samples

Samples from 76 patients were fully scanned with qPCR-HRM, comprising 62 patients with known variants (32 deleterious mutations, six polymorphisms, and 24 unclassified variants; Table 1), and 14 patients with a wild-type *MLH1* gene. For the variant names, the GenBank reference sequence NM_000249.2 was used. Nucleotide numbering reflects cDNA numbering, with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to HGVS guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

All the patients were fully screened with routine methods (prescreening by dHPLC, DNA sequencing, and MLPA). All the mutations were characterized by sequencing or MLPA.

The samples were obtained from three different French Hospitals: Institut Paoli Calmettes (Marseille, France), Hôpital Pitié Salpêtrière (Paris, France), and Centre René Huguenin (St Cloud, France). DNA was isolated from peripheral blood after obtaining the patients' specific informed consent for HNPCC genetic analysis.

DNA was extracted by column extraction with the QIAmp DNA blood kit or by magnetic particle technology with BioRobot EZ1 (both from Qiagen, Courtaboeuf, France). The quantity and quality of all experimental DNA samples were assessed with Nanodrop® technology (Coleman Technologies, Orlando, FL). DNA working solutions were prepared with an approximate concentration of 100 ng/µl. Then, the solutions were diluted in two steps to obtain a precise concentration of 4 ng/µl.

qPCR-HRM Conditions

Twenty primer pairs were designed for the *MLH1* gene (Supporting Table S1; available online at http://www.interscience.wiley.com/jpages/1059–7794/suppmat). Three primer pairs were designed for the coding sequences of the *ALB*, *ERBB2*, and *MET* genes as diploidy references. All *MLH1* primers were designed to obtain amplicons with a size of 256 bp on average (minimum: 195; maximum: 387). The three reference amplicons (*ALB*, *ERBB2*, and *MET*) were 139 bp, 219 bp, and 434 bp long, respectively, within the average sizes of *MLH1* amplicons. All were validated with a DNA concentration gradient (assays with 5, 10, 20, 40, and 80 ng of DNA in 15 µl final volume) to assess the efficacy of each primer pair. The selection criterion was a slope between −3.3 and −3.6; i.e., an efficacy from 90 to 100%. We also checked that the primers did not overlap any known single nucleotide polymorphisms (SNPs). The primers were designed to be annealed at the same temperature of 60°C. Primers for the reference and *MLH1* target exons (Supporting Table S1) were chosen with the assistance of the OLIGO6® (Molecular Biology Insights, Cascade, CO) and PRIMER3® software (http://fokker.wi.mit.edu/primer3/input.htm).

The qPCR and HRM were performed in a single run on a LightCycler 480® (Roche Diagnostics, Penzberg, Germany) in a reaction mix containing 20 ng of genomic DNA, 0.4 µM of each primer, and 3 mM $MgCl_2$ in the LightCycler 480 High Resolution

Table 1. Tested Mutations in the qPCR-HRM Approach*

Amplicon	Nucleotide variant NM_000249.2	Protein consequence NP_000240.1	Pathogenicity
Amplicon 1	c.37G>T	p.Glu13X	DEL
	c.65G>C	p.Gly22Ala	POL
	c.67delG	p.Glu23LysfsX13	DEL
Amplicon 2	c.117-16C>T	Intron	UV
	c.117-1G>C	Splice defect	DEL (splice defect)
	c.121G>C	p.Asp41His	UV
	c.174G>T	p.Leu58Phe	UV
	c.180G>A	Silent	POL
	c.198C>T	Silent	UV
	c.199G>C	p.Gly67Arg	DEL
	Duplication exons 2 to 3 (c.117_306dup)[b]	Exon duplication	DEL (exon duplication)
Amplicon 3	c.293_304 del12	p.Gly98_Gly101del	DEL
	c.298C>T	p.Arg100X	DEL
	c.302G>A	p.Gly101Asp	UV
Amplicon 4	c.350C>T	p.Thr117Met	UV
	c.375A>G	Silent	UV
	c.380+27A>G	Intron	UV
	Duplication exon 4 (c.307_380dup)[b]	Exon duplication	DEL (exon duplication)
Amplicon 5	c.381-33A>G	Intron	UV
	c.397G>T	p.Gly133X	DEL
	c.453+1G>T	Splice defect	DEL (splice defect)
Amplicon 6	c.454-60C>G	Intron	POL
	c.454-51T>C	Intron	POL
	c.474C>T	Silent	POL
	Duplication exons 6 to 8 (c.454_677dup)[b]	Exon duplication	DEL (exon duplication)
Amplicon 7	c.574_580+2del17	Splice defect	DEL (splice defect)
	c.588+2T>C	Splice defect	DEL (splice defect)
Amplicon 8	c.655A>G	p.Ile219Val	UV
	c.676C>T	p.Arg226X	DEL
Amplicon 9	c.702G>A	Silent	POL
	c.790+1G>A	Splice defect	DEL
	c.790+3A>T	Splice defect	DEL
Amplicon 10	c.793C>T	p.Arg265Cys	UV
	c.813delC	p.Leu272X	DEL
Amplicon 11	c.999del	p.Lys333AsnfsX34	DEL
	c.1037A>G	p.Gln346Arg	UV
Amplicon 12	c.1039-8T>A[a]	Intron	UV
	c.1039-15_22del8	Intron	UV
	c.1174_1175insGA	p.Lys392ArgfsX10	DEL
	c.1217G>A	p.Ser406Asn	UV
	c.1376C>G	p.Ser459X	DEL
	c.1408A>T	p.Arg470X	DEL
	c.1409+42T>A	Intron	UV
Amplicon 13	c.1558+14G>A	Intron	UV
	c.1558+1G>T	Splice defect	DEL (splice defect)
	Deletion exon 13 (c.1410_1558del)[b]	Exon deletion	DEL (exon deletion)
Amplicon 14	c.1616C>A	p.Ala539Asp	UV
	c.1624C>T	p.Gln542X	DEL
Amplicon 15	c.1668-19A>G	Intron	UV
	c.1731G>A	Splice defect	DEL (splice defect)
	c.1731+4A>G	Intron	UV
Amplicon 16	c.1852_1854delAAG	p.Lys618del	UV
	c.1754T>G	p.Leu585Arg	UV
	c.1857dupT	p.Glu620X	DEL
Amplicon 17	c.1941delG	p.Pro649LeufsX12	DEL
	c.1959G>T	Silent	UV
	c.1975C>T	p.Arg659X	DEL
	Deletion exons 17 to 19 (c.1897_2271del)[b]	Exons deletion	DEL (exon deletion)
Amplicon 18	c.2042dupC	p.Met682TyrfsX12	DEL

TABLE 1. Continued

Amplicon	Nucleotide variant NM_000249.2	Protein consequence NP_000240.1	Pathogenicity
Amplicon 19	c.2136G>A	p.Trp712X	DEL
	c.2146G>A	p.Val716Met	UV
	c.2190delT	p.Pro731LeufsX52	DEL

*Data are from 62 samples from: Centre René Huguenin, St. Cloud; Institut Paoli Calmettes, Marseille; and Hôpital Pitié Salpêtrière, Paris. The GenBank reference sequences used is NM_000249.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to HGVS guideline (www.hgvs.org/mutnomen). The initiation codon is codon 1.
[a]Only detected by direct sequencing.
[b]Large rearrangements.
DEL, deleterious mutation; UV, unclassified variant; POL, polymorphism.

Melting Master® containing ResoLight® dye (Roche Diagnostics) with PCR-grade water adjusted to a total volume of 15 µl.

The reaction conditions included an activation step at 95°C for 10 min followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 25 s. Before the HRM step, the products were heated to 95°C for 1 min and frozen to 40°C for 1 min. HRM was carried out over the range from 65°C to 95°C, rising at 1°C per second with 25 acquisitions per degree. All reactions were performed in replicate (duplicate or triplicate) in 384-well microtiter plates.

qPCR-HRM Analysis

Upon completion of the run, qPCR-HRM analysis was performed using the LightCycler 480 software (LC480; Roche) and an in-house written Microsoft Excel® (Redmond, WA) macro.

In qPCR analysis, some large rearrangements were directly detected by looking at the amplification curves. To gain further precision, we used the computational approach described below. The crossing point (Cp), is defined as the fractional cycle number at which the fluorescence generated by Resolight® dye with amplicon complex formation passes above background baseline computed automatically by LC480 software, according to the manufacturer's manuals. Cp is used as a quantitative measurement of the input target. It decreases linearly as a function of the log of input target quantity.

A precise amount of genomic DNA was added to each reaction mix. To reduce fluctuations in its quantity and quality, references were studied either within the gene by normalization with another *MLH1* exon result, or outside the gene by normalization with other genes, in this case the *ALB*, *ERBB2*, and *MET* genes. The Cp results from all the amplicons were used in qPCR analysis, except from the exon 12 in which only amplicon 12.2 was used in the quantitative approach.

Data were exported to a Microsoft Excel spreadsheet to calculate ratios with the $2^{-\Delta\Delta Cp}$ method [Bieche et al., 1998]. The formula used is $N_{ex} = 2^{-\Delta\Delta Cp}$, where the $\Delta\Delta$Cp value of the sample was determined by subtracting the average Cp value of the target exon from the maximum average Cp from other samples for this target exon. An N_{ref} value was also computed with Cp results from one of the references. A ratio was then computed by divided N_{ex} by N_{ref}. This ratio was normalized to 1 by dividing by the average ratio from the 14 wild-type samples. Therefore, a normal sample has a normalized N_{ex} close to 1. The thresholds were $N_{ex} < 0.75$ for deletions and $N_{ex} > 1.25$ for duplications (MLPA conditions).

For HRM, the melting curves must be normalized and the temperature shifted (temp-shifted) to make samples directly comparable. Modified curves can be obtained with LC480 software in the gene-scanning module (version 1.3; Roche). The normalized and temp-shifted melting curves correspond to the final curve after the normalization process. A mutated amplicon appears as a normalized and temp-shifted melting curve with a shape different from that of a wild-type amplicon.

The normalized and temp-shifted difference plot is obtained by deriving the melting profile of the sample and comparing it to the wild-type profile. This increase the variations signal in a mutated amplicon. When the melting curve had several melting domains, the analysis was performed in two steps, first studying the overall amplicon and then each domain. Sensitivity was set by default to 30% for all the amplicons. Between several wild-type amplicons, the normalized and temp-shifted difference plot varies within a range due to small random differences. Then, a range of normality can be determined for each amplicon—usually a relative signal difference between −2 and +2. Between a wild-type amplicon and a putative mutated amplicon, the normalized and temp-shifted difference plots have systematic differences for specific temperatures, which fall outside the range of normality. Traditionally, the differences were judged significant if the curves of a putative mutated amplicon were found with similar values outside the range of normality. To extent this, we considered in this study that replicates of a putative mutated amplicon must have similar patterns and be different from those of the wild-type samples. Thus, in this case, they were sequenced even when they lay within the range of normality.

Sequencing

Sequencing was used to confirm and characterize affected exons identified in HRM. The PCR products were analyzed on agarose gel and directly sequenced in both directions by using each PCR primer with the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems [ABI], Foster City, CA). There was no purification step before the sequencing reaction. Primers were used with a final concentration of 2.4 µM in a final volume of 10 µl. The cycling conditions consisted of 25 cycles at 96°C for 30 s, 50°C for 15 s, and 60°C for 2 min. The products of the sequencing reactions were cleaned up using Sephadex™ G-50 in a MultiScreen®-HV 96-well filter plate (Millipore, Billerica, MA). After purification, the sequences were determined in an ABI Prism 3130 automated sequencer.

Array-CGH

To confirm and characterize large rearrangements of the *MLH1* gene, a zoom-in CGH-array was used. An 11,000-oligonucleotide microarray was specially designed with home-designed oligonucleotides and with validated oligonucleotides (Agilent Technolo-

gies, Santa Clara, CA). Of these, 9,294 were located throughout the genome (1,481 Agilent oligonucleotides and 7,813 home-designed oligonucleotides on various other cancer predisposition genes), while 1,031 oligonucleotides were specifically home-designed and dedicated for the *MLH1* gene and its flanking regions. The analytical approach has been described elsewhere [Rouleau et al., 2007]. For the interpretation of the oligonucleotides signal, the chosen threshold was deleted if the $\log_2$ ratio was <-0.4 and duplicated if >0.4.

Results

Effective primer design is an important component of qPCR-HRM analysis. The performance (Cp, efficacy, number of domains, and range of normality) for each amplicon varied as described in Table 2. For qPCR, the typical Cp value was between 25 and 26 and efficacy was between 90% and 100%. For HRM, the maximum of the observed range of normality was between +3 and −3 in the normalized and temp-shifted difference plots for wild-type amplicons. There were less than two domains per amplicon. All the amplicons contained at least 35 nucleotides in the intron.

Large Rearrangements

All five complex mutations were detected by qPCR with the algorithm and threshold described (Fig. 1): duplication in exons 6 to 8, duplication in exons 2 to 3 (insufficient DNA to analyze all the exons), duplication in exon 4, and deletion in exon 17 to 19. Three examples of amplification curves are also given for a deletion in exon 13, a deletion in exon 17, and a duplication in exon 4 (Fig. 2).

Three complex mutations were confirmed with the dedicated *MLH1* CGH-array (Fig. 3). The breakpoints for the deletion of exon 4 were sequenced and the size was exactly 1,665 bases (chr3: 37019776–37021440 in hg18 nomenclature) duplicated without inversion. The duplication of exons 6 to 8 in the *MLH1* gene had an estimated size of between 5 and 6 kb. The deletion of exon 13 in the *MLH1* gene had an estimated size of ~3.5 kb.

In a DNA from a putative wild-type patient, a deletion was found in amplicon 19 (Fig. 1F) whereas the MLPA result was normal for this patient. New primers were selected and the unclassified variant c.*35_37del was found in the position of the first reverse primer, explaining the allele dropout. With the new primer pair for amplicon 19, no false positives were detected in the other 13 wild-type samples, tested in triplicate. This variant was not found by dHPLC and only one allele was then amplified.

Point Mutations

All the point mutations detected by dHPLC were clearly detected by HRM with the LC480 software algorithm (Table 1). There was no amplicon with false-positive results in the 14 wild-type patients.

Several of the results are illustrated in Figure 2. In exon 13, the mutation c.1558+1G>T and the variant c.1558+14G>A were clearly detected in both representation curves. It was possible to distinguish these two variants in the normalized and temp-shifted difference plot (Fig. 2A).

In exon 17, the mutation c.1941delG, the variant c.1959G>T, and the mutation c.1975C>T were clearly detected but were not distinguishable in the profile in normalized and temp-shifted melting curves or in the normalized and temp-shifted difference plots (Fig. 2B).

In exon 4, the variant c.380+27A>G was detected in the normalized and temp-shifted difference plots. Due to the position of the mutation at the end of the amplicon, the signal magnitude was close to that of the reference curve and was very low, with a

Table 2. Quantitative and Qualitative Data for Primers Obtained From Concentration Gradient (Assays 5, 10, 20, 40, and 80 ng/μl) and From Sample Assays (N = 76).

	qPCR			HRM		
Amplicons *MLH1*	Average Cp	Efficacy (%)	Domain (D domain)	Range of normality	Intron in 5′	Intron in 3′
1	25	88	1D	−0.5 to 1.8	−73	+48
2	26	91	2D	−2 to 2.5	−87	+72
3	24	98	1D	−1.2 to 1.9	−77	+147
4	25	100	2D	−2 to 3	−70	+73
5	29	98	2D	−2 to 2	−67	+55
6	25	95	1D	−0.5 to 1	−85	+87
7	25	94	1D	−1 to 1.5	−72	+88
8	27	100	2D	−1.5 to 3	−81	+85
9	25	93	2D	−1.3 to 1.5	−102	+53
10	26	89	1D	−1.2 to 2.7	−80	+65
11	26	91	2D	−3 to 1.8	−51	+36
12–1	30	—	1D*	−2 to 2	−77	—
12–2	25	95	2D	−1 to 1.2	/	+102
13	25	89	1D	−0.5 to 2	−124	+61
14	24	100	1D	−0.8 to 1.5	−100	+46
15	25	88	1D	−1.5 to 1.5	−71	+65
16	25	91	1D	−1.5 to 1.5	−52	+59
17	25	94	1D	−1.3 to 2	–110	+60
18	25	89	2D	−1.5 to 1.5	–71	+58
19	26	93	2D	−1.5 to 2	–71	+92
ALB	**25**	**98**	—	—		
ERBB2	**24**	**90**	—	—		
MET	**26**	**89**	—	—		

*Amplicon 12.1: one domain, but a initial low melting temperature associated to a T-stretch.

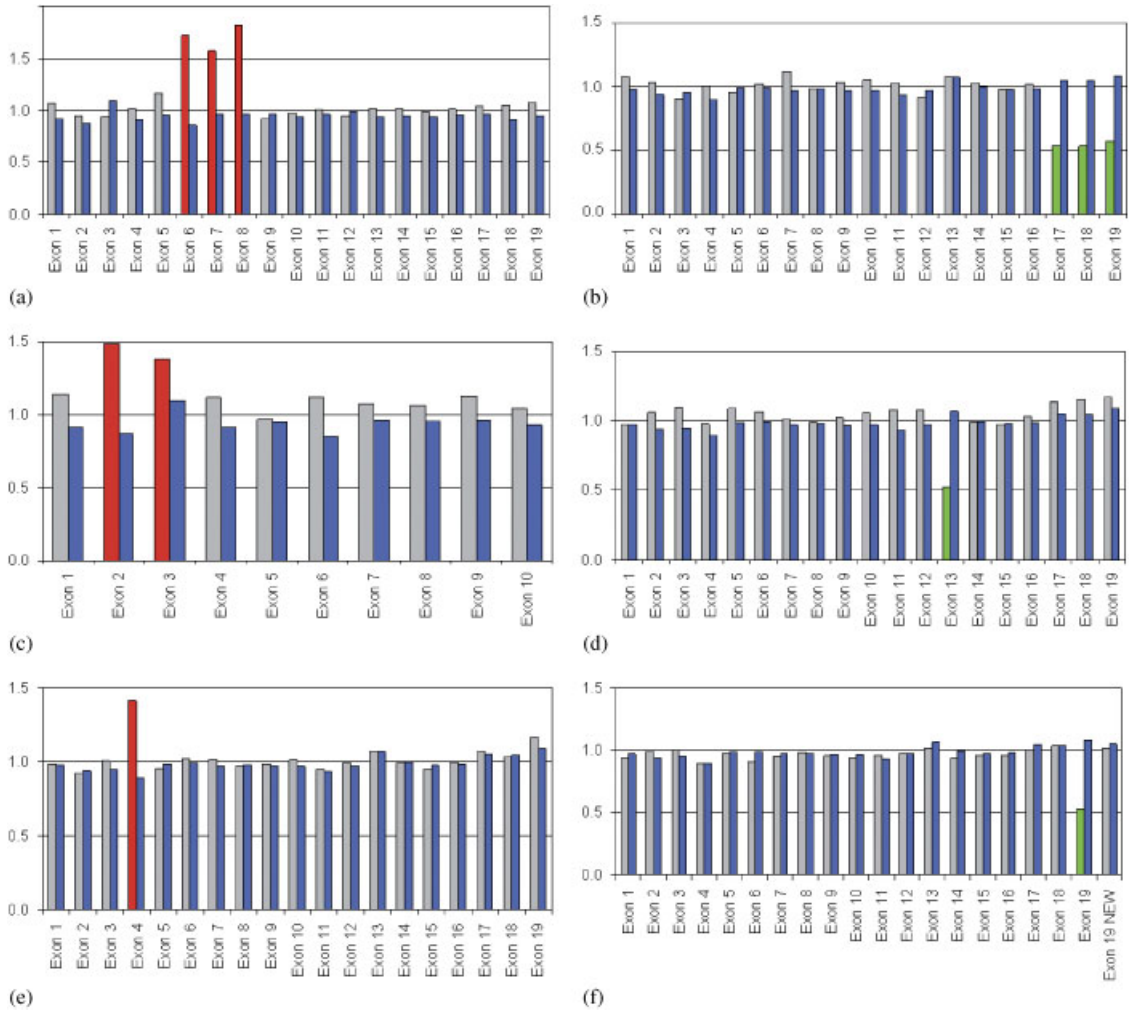


Figure 1. qPCR analysis for each *MLH1* amplicon in normalized N_{ex} to a reference gene (*ALB*, *ERBB2*, and *MET*). The left gray box is a control and the right blue box represents a patient with a deletion (in green) or a duplication (in red). **A**: Duplication of exons 6 to 8. **B**: Deletion of exons 17 to 19. **C**: Duplication of exons 2 to 3 (insufficient DNA to analyze the other exons). **D:** Deletion of exon 13. **E:** Duplication of exon 4. **F**: False deletion of exon 19 due to a mutation (c.*35_37del) in the reverse primer, confirmed by sequencing. The new primers "exon 19 NEW" confirmed the absence of large rearrangements.

relative signal difference below 1.5. The relative signal difference plotted against a wild-type curve was clearly distinguishable thanks to the duplicate (Fig. 2C).

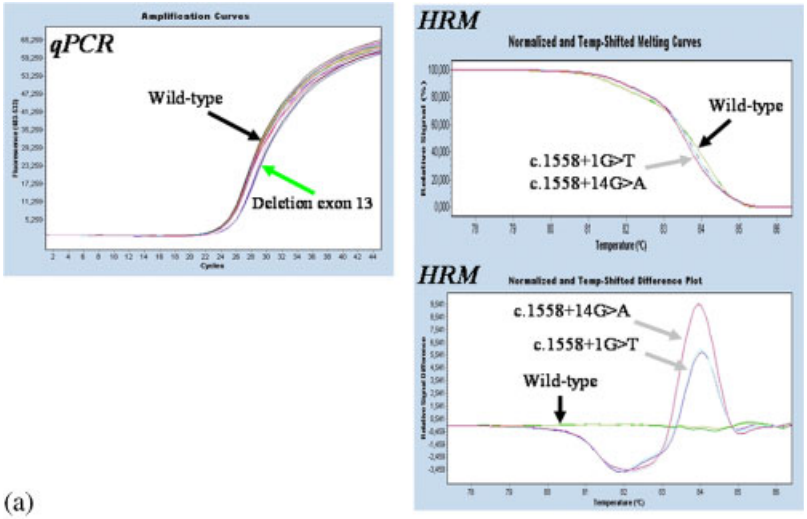
Two amplicons had limited performance and several redesigns failed to improve them (Table 2). Amplicon 12-1 had a Cp close to 30, suggesting high variability in PCR efficiency. The forward primer is close to a stretch of 20 T, which can generate a background noise and lower the signal of variants in this region. Variant c.1039–8T>A was not detected by qPCR-HRM or by dHPLC. This variant is close to the T stretch and also lies in a low melting temperature region. All the other mutations and variants were successfully detected (c.1039–15_22del8, c.1217G>A, and c.1174_1175insGA). We conclude that it is necessary to systematically sequence amplicon 12-1 in qPCR-HRM products. Amplicon 5 also had a Cp close to 30, but even here the mutations and variants c.381–33A>G, c.397G>T, and c.453+1G>T were validated.

Discussion

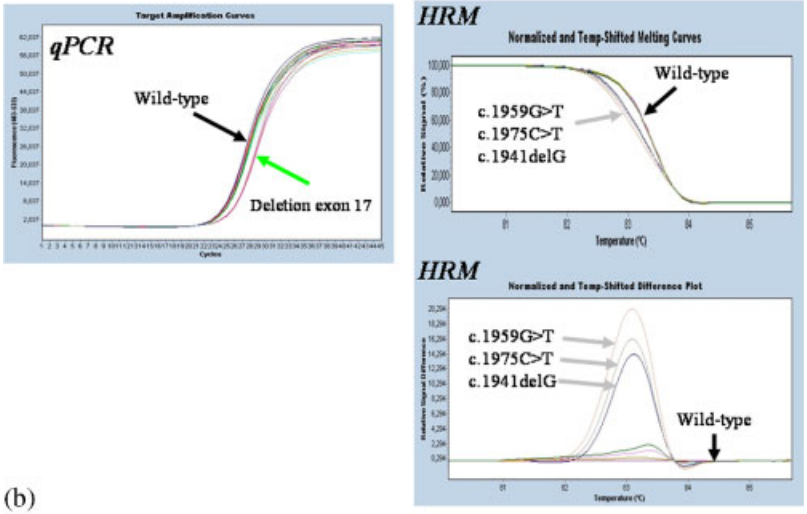
There are numerous methods for detecting gene mutations, each with its own advantages and drawbacks. Prescreening methods such as dHPLC are advantageous in that they reduce the amount of sequencing and avoid the sequencing of wild-type amplicons. The aim of this study was to validate an approach, named "qPCR-HRM" combining qPCR and HRM to prescreen for both point mutations (HRM) and large rearrangements (qPCR gene dosage) in a single run. We found this approach to be both versatile and sensitive.

Relative to dHPLC+MLPA, the main differences are the use of replicates, the simultaneous reading of 384 points, the quantitative information, and the absence of post-PCR manipulations.

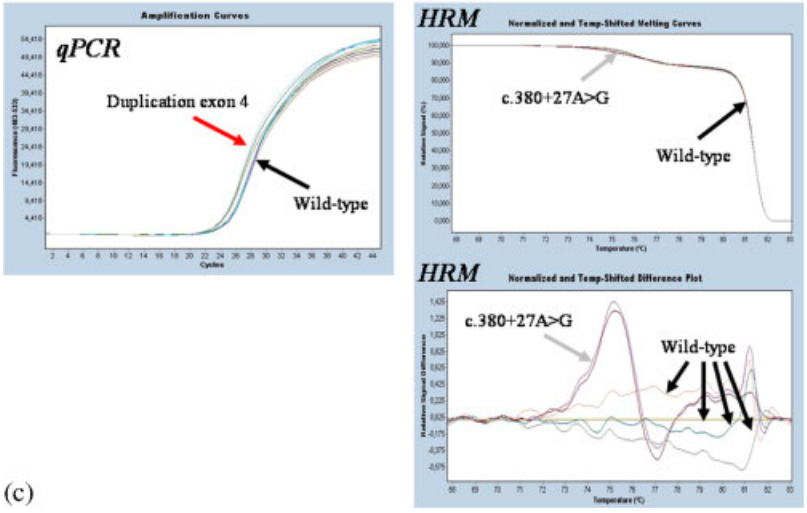
First, replicates are not, traditionally, mandatory to obtain HRM data to screen for point mutations. We recommend them because triplicate data is helpful to quantify a target exon [Bieche et al., 1998]. In case of an error in one PCR assay, two other measurements are available. For HRM curves, measurement in replicate improves confidence in the selection of putative mutated amplicons. Indeed, if the replicate curves are similarly different from a series of normal samples, a mutation can be suspected even if the intensity is within the range of normality. In case of mutations at the end of an amplicon (intronic variants), replicate data are highly valuable, as shown for exon 4 (c.380+27A>G) in Figure 2C. For this specific variant, we confirmed the ability of the



(a)



(b)



(c)

Figure 2. **A**: Exon 13 of the *MLH1* gene. Left side: qPCR amplification curve with deletion of exon 13. Right side: HRM curves with the mutations c.1558+1G>T and c.1558+14G>A in normalized and temp-shift melting curve and difference plot (duplicate). **B**: Exon 17 of the *MLH1* gene. Left side: qPCR amplification curve with deletion of exon 17. Right side: HRM curves with the mutations c.1941delG, c.1959G>T, and c.1975C>T normalized and temp-shifted melting curves (duplicate) and difference plot (singleton). **C**: Exon 4 of the *MLH1* gene. Left side: qPCR amplification curve with a duplication of exon 4. Right side: HRM curves with the mutations c.380+27A>G in normalized and temp-shifted melting curves and difference plot (duplicate). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

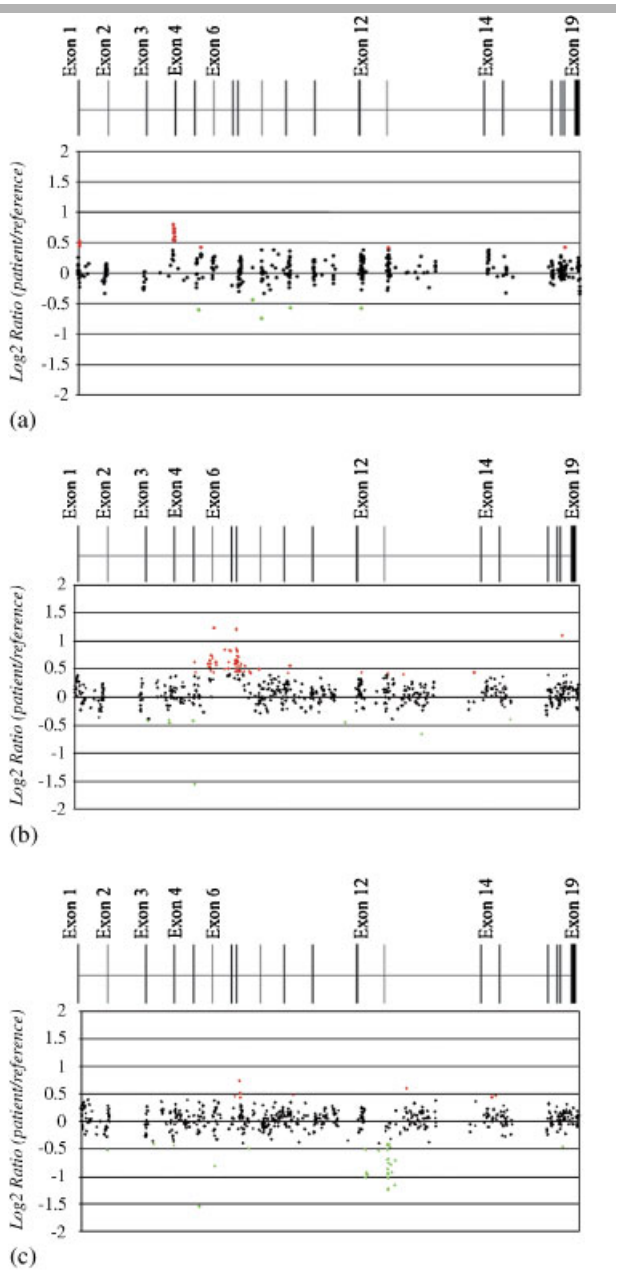


Figure 3. Dedicated zoom-in array-CGH results with the position of exons above and the $\log_2$ ratio of intensity for each oligonucleotide. **A**: Duplication of exon 4 in the *MLH1* gene, precise size (sequenced) 1,665 bp. **B**: Duplication of exons 6 to 8 in the *MLH1* gene with an estimated size between 5 and 6 kb. **C**: Deletion of exon 13 in the *MLH1* gene with an estimated size of around 3.5 kb. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

approach to detect it in a blind study of 55 samples in triplicate (data not shown).

Second, in qPCR-HRM, one 384-plate can be used to screen five patients with 20 *MLH1* amplicons and three references in triplicate. The same approach with dHPLC would require 345 individual tubes and as many or even more runs. The qPCR-HRM approach can also benefit from the use of a sample distribution system to simplify the filling of 384 wells.

Moreover, the quantitative information can give direct information on the number of amplified alleles. If a primer hybridizes on a mutation, for example a nucleotide substitution or deletion/duplication, the allele bearing this variant will not be amplified, thus suggesting an exon deletion. This information helps to identify variants or mutations within the primers. Moreover, a putative deleterious mutations in the allele could be linked to this variant in the primer. Other methods will be used to confirm the allele dropout, such as semiquantitative PCR or dedicated array-CGH.

HRM has been shown to be comparable to dHPLC in terms of its sensitivity and specificity [Chou et al., 2005]. In replicate, the sensitivity and specificity of qPCR-HRM were found identical in this study to those of dHPLC+MLPA. All the 62 mutations were found (100% of sensitivity in comparison to dHPLC+MLPA). There was one false positive in qPCR and none in HRM (99.8% of specificity in comparison to dHPLC+MLPA). So, the replicate approach should not require any extra sequencing in comparison to dHPLC+MLPA. Like dHPLC, HRM is of limited use for detecting mutations that only weakly modify the melting temperature of the amplicon, as we showed for exon 12-1. qPCR-HRM cannot be used to genotype variants, as we showed with the three variants of exon 17 in Figure 2B. Both dHPLC and HRM are prescreening methods and neither can completely eliminate the need for sequence confirmation. One major advantage of qPCR-HRM is that there are no post-PCR manipulations, which can increase the risk of error or contamination. The other key advantage is to provide one-step prescreening for point mutations and large rearrangements.

Contrary to other semiquantitative methods, the amplification curve provides a rapid estimation of DNA quality. In MLPA and QMPSF, post-PCR manipulations are needed for fragment analysis, which also requires fluorescently labeled primers. Multiplexing is the main advantage of these methods. However, in gene screening, it is necessary to PCR-amplify all the amplicons, even if a direct sequencing strategy is chosen. In the overall process, qPCR-HRM avoids the need for specific and separate PCR assays for large rearrangements.

When using 15-µl final volumes, in triplicate and in 384-well plates, the reagent cost of *MLH1* gene screening in qPCR-HRM is similar to that of dHPLC+MLPA (in singleton) per DNA sample. However, qPCR-HRM outperforms current methods in salary and maintenance costs by its rapidity, simple sample manipulation, low maintenance requirements, the absence of daily checking, and rapid optimization process. Indeed, qPCR reactions and HRM are performed in a single run in 90 min without post-PCR manipulation.

DNA sequencing is considered as a gold standard for the *MLH1* screen in many laboratories. However, sequencing takes a long time to obtain and analyze the results. The rapidity and low cost of prescreening methods is their main asset. The qPCR-HRM approach obviates the need for an automated capillary sequencer in the prescreening process and limits its use to final validation of exons flagged by this method. Because the total consumption of primers can be followed with the amplification curve, there is no need for purification before the sequencing reaction. Sequencing and array-CGH provide the final measurements for the detection, confirmation, and characterization of variants.

Three reference genes were used here, with a size ranging from 139 bp to 434 bp. The results clearly showed that larger size was associated with lower efficiency. The use of several sizes can help to indicate the level of DNA quality, as larger amplicons are also more sensitive to DNA degradation. This approach has been proposed to validate the quality of DNA after formalin-fixed and paraffin-embedded (FFPE) tissue extraction [van Beers

et al., 2006]. For Cp normalization an *MLH1* amplicon can also be used as reference. It is necessary however to have at least one reference to detect any whole-gene deletion or duplication. In this study, we observe no variation due to the DNA extraction methods between samples from the three hospitals. All the Cp results were homogeneous for each amplicon tested. Nevertheless, it should be important to use a series with a homogeneous extraction process to reduce variations. DNA quantification is the crucial point. Indeed, in HRM analysis, variations in DNA quantification can lead to false positives. For each experiment in qPCR-HRM, DNA quality can be questioned when there are Cp results systematically outside the usual range for several exons, a final amplification curve under the average level, and a low initial level of fluorescence for the melting curve.

The qPCR-HRM approach requires careful attention to the design of primers and amplicons. An optimal primer will yield an amplicon size of less than 300 bp, a qPCR efficiency greater than 90%, a standard deviation in triplicate close to 0.2, and a low range of normality. Ideally, Cp values should be similar for all amplicons (here around 25). Two amplicons were particularly difficult to design and obtain a correct Cp result. For the exon 12 amplicon, there were two sets of primers. The first, in the 5′ part of the exon, had a high Cp close to 30. A stretch of 20T could limit the detection of mutations. This amplicon was directly sequenced. We propose the use of two sets of primers: one for HRM covering the entire exon, and another for quantitative PCR covering part of the same exon.

A larger study is underway to confirm the sensitivity and specificity of this approach in routine practice and to extend it to the other *MMR* genes. For *MSH2* and *MSH6*, the main difficulties will be the presence of several amplicons with stretch of nucleotides, a higher proportion of A/T in *MSH2*, and the existence of numerous polymorphisms in *MSH6* (exons 1, 2, 3, 7, and 8).

Conclusion

We describe a rapid, highly sensitive, inexpensive, high-throughput qPCR-HRM method to prescreen for point mutations and large rearrangements in the *MLH1* gene. This new and reliable method can be used to detect point mutations and large rearrangements in a single run prior to characterization by sequencing or array-CGH. It can thus reveal deleterious mutations rapidly in a large series of samples.

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References

Beck NE, Tomlinson IP, Homfray T, Frayling I, Hodgson SV, Harocopos C, Bodmer WF. 1997. Use of SSCP analysis to identify germline mutations in HNPCC families fulfilling the Amsterdam criteria. Hum Genet 99:219–224.

Bieche I, Olivi M, Champeme MH, Vidaud D, Lidereau R, Vidaud M. 1998. Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer. Int J Cancer 78:661–666.

Charbonnier F, Olschwang S, Wang Q, Boisson C, Martin C, Buisine MP, Puisieux A, Frebourg T. 2002. MSH2 in contrast to MLH1 and MSH6 is frequently inactivated by exonic and promoter rearrangements in hereditary nonpolyposis colorectal cancer. Cancer Res 62:848–853.

Chou LS, Lyon E, Wittwer CT. 2005. A comparison of high-resolution melting analysis with denaturing high-performance liquid chromatography for mutation scanning: cystic fibrosis transmembrane conductance regulator gene as a model. Am J Clin Pathol 124:330–338.

de Juan I, Esteban E, Palanca S, Barragan E, Bolufer P. 2008. High-resolution melting analysis for rapid screening of BRCA1 and BRCA2 Spanish mutations. Breast Cancer Res Treat. (Online publication)

De Leeneer K, Coene I, Poppe B, De Paepe A, Claes K. 2008. Rapid and sensitive detection of BRCA1/2 mutations in a diagnostic setting: comparison of two high-resolution melting platforms. Clin Chem 54:982–989.

Gerhardus A, Schleberger H, Schlegelberger B, Gadzicki D. 2007. Diagnostic accuracy of methods for the detection of BRCA1 and BRCA2 mutations: a systematic review. Eur J Hum Genet 15:619–627.

Holinski-Feder E, Muller-Koch Y, Friedl W, Moeslein G, Keller G, Plaschke J, Ballhausen W, Gross M, Baldwin-Jedele K, Jungck M, Mangold E, Vogelsang H, Schackert HK, Lohsea P, Murken J, Meitinger T. 2001. DHPLC mutation analysis of the hereditary nonpolyposis colon cancer (HNPCC) genes hMLH1 and hMSH2. J Biochem Biophys Methods 47:21–32.

Kurzawski G, Suchy J, Lener M, Klujszo-Grabowska E, Kladny J, Safranow K, Jakubowska K, Jakubowska A, Huzarski T, Byrski T, Debniak T, Cybulski C, Gronwald J, Oszurek O, Oszutowska D, Kowalska E, Gozdz S, Niepsuj S, Slomski R, Plawski A, Lacka-Wojciechowska A, Rozmiarek A, Fiszer-Maliszewska L, Bebenek M, Sorokin D, Sasiadek MM, Stembalska A, Grzebieniak Z, Kilar E, Stawicka M, Godlewski D, Richter P, Brozek I, Wysocka B, Limon J, Jawien A, Banaszkiewicz Z, Janiszewska H, Kowalczyk J, Czudowska D, Scott RJ, Lubinski J. 2006. Germline MSH2 and MLH1 mutational spectrum including large rearrangements in HNPCC families from Poland (update study). Clin Genet 69:40–47.

Lynch HT, de la Chapelle A. 1999. Genetic susceptibility to non-polyposis colorectal cancer. J Med Genet 36:801–818.

Rouleau E, Lefol C, Tozlu S, Andrieu C, Guy C, Copigny F, Nogues C, Bieche I, Lidereau R. 2007. High-resolution oligonucleotide array-CGH applied to the detection and characterization of large rearrangements in the hereditary breast cancer gene BRCA1. Clin Genet 72:199–207.

Sevilla C, Julian-Reynier C, Eisinger F, Stoppa-Lyonnet D, Bressac-de Paillerets B, Sobol H, Moatti JP. 2003. Impact of gene patents on the cost-effective delivery of care: the case of BRCA1 genetic testing. Int J Technol Assess Health Care 19:287–300.

Staaf J, Torngren T, Rambech E, Johansson U, Persson C, Sellberg G, Tellhed L, Nilbert M, Borg A. 2008. Detection and precise mapping of germline rearrangements in BRCA1, BRCA2, MSH2, and MLH1 using zoom-in array comparative genomic hybridization (aCGH). Hum Mutat 29:555–564.

Takano EA, Mitchell G, Fox SB, Dobrovic A. 2008. Rapid detection of carriers with BRCA1 and BRCA2 mutations using high resolution melting analysis. BMC Cancer 8:59.

van Beers EH, Joosse SA, Ligtenberg MJ, Fles R, Hogervorst FB, Verhoef S, Nederlof PM. 2006. A multiplex PCR predictor for aCGH success of FFPE samples. Br J Cancer 94:333–337.

Wahlberg S, Liu T, Lindblom P, Lindblom A. 1999. Various mutation screening techniques in the DNA mismatch repair genes hMSH2 and hMLH1. Genet Test 3:259–264.

Wang Y, Friedl W, Lamberti C, Jungck M, Mathiak M, Pagenstecher C, Propping P, Mangold E. 2003. Hereditary nonpolyposis colorectal cancer: frequent occurrence of large genomic deletions in MSH2 and MLH1 genes. Int J Cancer 103:636–641.

Wijnen J, Vasen H, Khan PM, Menko FH, van der Klift H, van Leeuwen C, van den Broek M, van Leeuwen-Cornelisse I, Nagengast F, Meijers-Heijboer A, Lindhout D, Griffioen G, Cats A, Kleibeuker J, Varesco L, Bertario L, Bisgaard M, Mohr J, Fodde R. 1995. Seven new mutations in hMSH2, an HNPCC gene, identified by denaturing gradient-gel electrophoresis. Am J Hum Genet 56:1060–1066.