Methods

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



Human Mutation

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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

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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.

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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

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 $100 \text{ ng/}\mu$ l. 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 $480^{(B)}$ (Roche Diagnostics, Penzberg, Germany) in a reaction mix containing 20 ng of genomic DNA, $0.4 \,\mu$ M of each primer, and 3 mM MgCl₂ in the LightCycler 480 High Resolution

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

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Amplicon 4c.350C >Tr.The 117 MerUVc.375A >GSlentUVc.380 + 27A > GIntronUVDuplication exon 4Exon duplicationUV(c.307 - 380 dup)*Exon duplicationUVamplicon 5c.381 - 33A > GIntronUV(c.307 - 380 dup)*Splice defectDEL (exon duplicationAmplicon 6c.454 + 60C > GIntronPOL(c.454 + 571 > CSplice defectDEL (splice defect)Duplication exons 6 to 8Exon duplicationPOL(c.454 + 571 and p)*Splice defectDEL (splice defect)Amplicon 7c.574 - 580 + 2del17Splice defectDEL (splice defect)Amplicon 8c.656 > GSplice defectDEL (splice defect)(c.454 - 677 dup)*Splice defectDEL (splice defect)Amplicon 9c.70G > ASplice defectDEL(c.454 - 677 dup)*Splice defectDELAmplicon 10c.73G > ASplice defectDEL(c.454 - 677 dup)*Splice defectDELAmplicon 10c.73G > ASplice defectDEL(c.1037 A > Gp.14225XDELDEL(c.1037 A > Gp.14235A anfX5X4DEL(c.1037 A > Gp.14335A anfX5X4DEL(c.1037 A > Gp.14337A anfX5X4DEL(c.1037 A > Gp.14337A anfX5X4DEL(c.1037 A > Gp.143747AIntronUV(c.1358 + 112 > Tp.14337AnfX5X4DEL(c.1364 AT > AIntronUV				
1.373 > GSilentUVG380 + 27.8 > GIntronUVDuplication exon 4Exon duplicationDEL (exon duplication(c307, 380dup)*IntronUVc3973 > Gp.Gly133XDEL(c435+1G>Tp.Gly133XDEL(c435+1G>TSplice defectDEL (splice defect)Amplicon 6(c445+602 > GIntronPOL(c442 > TSubation 2000C444-517Splice defect)Duplication exons 6 to 8Exon duplicationDEL (splice defect)(c444 - 27 K)Splice defect)DEL (splice defect)(c444 - 57 K)Splice defect)DEL (splice defect)(c444 - 677 K)Splice defect)DEL (splice defect)(c444 - 677 K)Splice defectDEL (splice defect)(c588 - 27 S < p.Jacl 219Val	N 11 4		1 / 1	
3.80 ±7A>GIntronUVDuplication exon 4Exon duplicationDEL (exon duplication(c.307,380 dup) ⁶ IntronUV(c.307,380 dup) ⁶ GMDEL (exon duplication(c.307,65 TSplice defectDEL (splice defect)Amplicon 6(c.454,65 C-GIntronPOL(c.454,65 T-CSplice defectPOL(c.454,67 Lap)Splice defectDEL (splice defect)Diplication exons 6 to 8Exon duplicationDEL (splice defect)(c.454,677 Lap)Splice defectDEL (splice defect)Amplicon 7(c.574,580 + 2del17Splice defectDEL (splice defect)Amplicon 8(c.655A)>Gp.Hi219ValUV(c.794 - 5A)Splice defectDEL (splice defect)Amplicon 9(c.702G)>ASplice defectDEL (splice defect)(c.790 + 5A) ~ TSplice defectDELAmplicon 10(c.793C)>Tp.Jag265CysUV(c.813dECp.Jag333fX34)DELAmplicon 11(c.999dHp.Jus333fX34)DEL(c.1037A)>Gp.Jus337afX34DEL(c.1174,L175)mSGAp.Jus337gK310DEL(c.1174,L175)mSGAp.Jus337gK310DEL(c.1174,L175)mSGAp.Jus337gK310DEL(c.1174,L175)mSGAp.Jus334pCDEL (son defetion)(c.1174,L175)mSGAp.Jus334gK10DEL (son defetion)(c.1176,L27)p.Jus34pCUV(c.1176,L27)p.Jus34pCUV(c.1176,L27)p.Jus34pCDEL (son defect)	Amplicon 4		-	
Duplication exon 4 (c307_380dup)*Exon duplicationDEL (exon duplication (c307_580dup)*Amplicon 5C.381-33A-5G (C381-34A-5GIntronUV (c397G-5T(c307_65Tp.G[133XDEL (s]bice defect)DEL (splice defect)Amplicon 6C454-617-5C (c474C-5TIntronPOI (c474C-5T(c454-677dup)*DEL (splice defect)DEL (splice defect)Amplicon 7c.574_580+2del17Splice defectDEL (splice defect)(c454_677dup)*Splice defectDEL (splice defect)Amplicon 8c.655A-5Gp.He219ValUVAmplicon 9c.702G-5ASplice defectDEL (splice defect)(c700-71)p.Arg26SC/3SDEL (splice defect)DEL (splice defect)Amplicon 10c.7302-5ASplice defectDEL (splice defect)(c1037A) 5Gp.Jel272XDEL (c700+1G>ADEL (splice defect)DEL (splice defect)Amplicon 11c.999delp.Jus33Aanfx34DEL (UV(c103-7A) 5Gp.Ser459XDEL (splice defect)UV(c103-7A) 5Gp.Ser459XDEL (splice defect)DEL 				
(c.307_380dmp) ^b IntronUVAmplicon 5C381-333-SGIntronDELC381-333-SGglice defectSplice defectDELAmplicon 6C453+1G>TSplice defectPOIC454-5GIntronPOIC454-5TSilentPOIC474-2>TSilentPOIC474-2>TSilentPOIDiplication econs 6 to 8Econ duplicationPOIC474-2>TSplice defectDEL (splice defect)Amplicon 7C54_501-2del17Splice defectDEL (splice defect)Amplicon 8C655A>Gp.Hc219KalUVC700-13A>TSplice defectDELDEL (splice defect)Amplicon 9C702G>ASilentPOIC700-13A>TSplice defectDELDELAmplicon 10C702G>ASplice defectDELC700-13A>TSplice defectDELDELAmplicon 11C999delp.hys333Anfx34DELC107A>Gp.Gh346ArgUVUVAmplicon 12C1039-8T>A*IntronUVC107G>Ap.Arg470XDELDELC117G>ITSinsGAp.Lys392AnfsX14DELDELC117G>Ap.Arg470XDELSplice defectC117G>Ap.Arg470XDELSplice defectC117G>Ap.Arg470XDELSplice defectC117G>Ap.Arg470XDELSplice defectC117G>Ap.Arg470XDELSplice defectC117G>ASplice defectDEL (splice				
Amplicon 5c. 33.33.5 - GIntronUVc. 397G > Tp.Gly13XDELc. 453 + 16 > TSplice defectDEL (splice defect)Amplicon 6c. 454.5 + 67.6 GIntronPOLc. 474 - 5 TSilentPOLDuplication exons 6 to 8Exon duplicationDEL (splice defect)amplicon 7c. 574_580+2del17Splice defectDEL (splice defect)c. 654 - 677dup) ¹⁶ Splice defectDEL (splice defect)Amplicon 8c. 6555 > Gp.Hc219ValUVc. 790 + 3A > TSplice defectDELAmplicon 9c. 700 + 16 > ASplice defectDELc. 790 + 3A > TSplice defectDELAmplicon 10c. 790 + 3A > TSplice defectDELc. 1037 > Gp.Gly333AstnfX34DELAmplicon 11c. 999delp.Jcy327XDELc. 1039 - 8T > A ⁶ IntronUVc. 1039 - 8T > A ⁶ IntronUVc. 1039 - 15_2del8IntronUVc. 1039 - 15_2del8IntronUVc. 1174_1175insGAp.Jcy392ArgfsX10DELc. 1409 + 42T > AIntronUVc. 1409 + 42T > AIntronUV<			Exon duplication	DEL (exon duplication)
c.3970>Tp.G133XDELc.453+1G>TSplice defectDEL (oplice defect)Amplicon 6c.454+1G>TSplice defectDEL (oplice defect)c.454-51T>CIntronPOLc.474C>TSilentPOLDuplication exons fo to 8SilentDEL (exon duplicatioc.474C>TSplice defectDEL (splice defect)Amplicon 7c.574_580+2del17Splice defectDEL (splice defect)Amplicon 8c.655A_Gp.Hc219ValUVAmplicon 8c.675C>Tp.Ag226XDELc.7002>ASilentDELc.790+1G>ASplice defectDELc.790+1G>ASplice defectDELAmplicon 10c.793C>TSplice defectDELc.790+3A>TSplice defectDELc.1037A>Gp.G1346ArgUVc.1037A>Gp.G1346ArgUVc.1174_1175insGAp.Js332ArgfX10DELc.127G>Ap.Ser406ArgUVc.127G>Ap.Ser406ArgUVc.1409+42T>AIntronUVc.1409+42T>AIntronUVc.1558+14G>AIntronUVc.165C>Ap.Ag450XDEL (splice defect)c.166C>Ap.Ag450XDEL (splice defect)c.1616C>Ap.Ag450XDEL (splice defect)c.1616C>Ap.Ag450XDEL (splice defect)c.1616C>Ap.Ag450XDEL (splice defect)c.1731+A>GIntronUVc.1731+A>GIntronUVc.173G>ASplice def				
amplicon 6c.454-60C> GIntronDEL (splice defect)Amplicon 6c.454-60C> GIntronPOLc.444-51T> CIntronPOLDuplication econs 6 to 8Exon duplicationExon duplication(c.454_677aup) ¹⁰ Splice defectDEL (splice defect)c.574_580+2del17Splice defectDEL (splice defect)amplicon 7c.574_580+2del17Splice defectDEL (splice defect)amplicon 8c.658p.4222XDELamplicon 9c.702C>ASplice defectDELamplicon 10c.790+1G>ASplice defectDELamplicon 11c.999delp.4225XUVamplicon 12c.813delCp.42333AsnfsX34DELamplicon 12c.1037A>Gp.4333AsnfsX34DELamplicon 12c.1039-8T>A*IntronUVc.1174_1175insGAp.4ys30AsnfsX34DELamplicon 13c.1176_Ap.4ys470XDELc.1175_AGp.4ys470XDELc.1176_Ap.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AGp.4ys470XDELc.1176_AG	Amplicon 5	c.381-33A>G	Intron	UV
Amplicon 6 6454-60C>G Intron POL c454-50T>C Intron POL c474C>T Silent POL Duplication econs 6 to 8 Exon duplication POL c474C>T Silent POL Amplicon 7 C574_580+2del17 Splice defect DEL (econ duplicatio c588+2T>C Splice defect DEL (splice defect) Amplicon 8 c655A>G p.Ile219Val UV c588+2T>C Splice defect DEL Amplicon 9 c702G>A Silent POL c700+1G>A Splice defect DEL Amplicon 10 c793C>AT p.Arg26SCys UV c1037A>G p.Leu272X DEL Amplicon 11 c1037A>G p.Leu272X DEL c1039-15_22del8 Intron UV c1376C>G p.Serd50Kan UV c1408A>T intron <td></td> <td>c.397G>T</td> <td>p.Gly133X</td> <td>DEL</td>		c.397G>T	p.Gly133X	DEL
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$		c.453+1G>T	Splice defect	DEL (splice defect)
c474C>TSilentPOLDuplication exons 6 to 8Exon duplicationDEL (eson duplication(c454_c977dup) ¹⁰ Exon duplicationDEL (splice defect)c588+27>CSplice defectDEL (splice defect)c588+27>CSplice defectDEL (splice defect)Amplicon 8c655A>Gp.Ilce19ValUVc702G>ASilentDELc709+3A>TSplice defectDELc709+3A>TSplice defectDELc1037+3Cp.Grl3A6ArgUVc1037+3Cp.Gla9AfArgUVc1037+3CSplice defectDELc1037+3CSplice defectUVc1174_1175insGAp.Ser406AngUVc1376C>GSplice defectDELc1037+3CSplice defectDELc1376C>GSplice defectDELc1376C>GSplice defectDELc1376C>GSplice defectDELc1376C>GSplice defectDEL	Amplicon 6	c.454-60C>G	Intron	POL
Duplication exos 6 to 8 (c454_677dup)*Exon duplicationDEL (exon duplicationAmplicon 7c574_580+2del17Splice defectDEL (splice defect)8c655.5.4.5.6ple19ValUVc676C>Tp.Arg26XDELAmplicon 8c676C>TSplice defectDELc790+16.5.ASplice defectDELc790+16.5.ASplice defectDELc790+16.5.ASplice defectDELc790+16.5.Ap.Arg265CySUVc790+16.5.Ap.Leu272XDELAmplicon 10c.999delp.Leu272XDELc1037A>Gp.Lis333AnfX34DELc1037A>Gp.Lis333AnfX34DELc1037A>Gp.Ser406AsnUVc1174_1175insGAp.Ser406AsnUVc1174_1175insGAp.Ser498XDELc1376C>Gp.Ser498XDELc158+1G>Tp.Ser498AsnDELc158+1G>TSplice defectDEL (splice defect)Deletion exon 13c.1588+1G>TSplice defectc158+1G>TSplice defectDEL (splice defect)Deletion exon 13c.1616C>Ap.Ala539Aspc1731G>ASplice defectDEL (splice defect)c1731H>AIntronUVc1731H>ASplice defectDEL (splice defect)c1731H>ASplice defectDEL (splice defect)c1731H>ASplice defectDEL (splice defect)c1731H>ASplice defectDEL (splice defect)c1731H>ASplice defectDEL (splice defect) <t< td=""><td>I</td><td>c.454-51T>C</td><td>Intron</td><td>POL</td></t<>	I	c.454-51T>C	Intron	POL
		c.474C>T	Silent	POL
		Duplication exons 6 to 8	Exon duplication	DEL (exon duplication)
Amplicon 7c.574_580+2del17Splice defectDEL (splice defect)c.568+2T>CSplice defectDEL (splice defect)Amplicon 8c.655A > Gp.Jel219ValUVc.700C > ASilentP.Arg226XDELAmplicon 9c.702G > ASilent GefectDELc.790+1G > ASplice defectDELc.790+3A > TSplice defectDELc.813delCp.Leu272XDELAmplicon 10c.999delp.Jys33AsnfsX34DELc.813delCp.Gln346ArgUVAmplicon 12c.1037+5 A*IntronUVc.1039-8T > A*IntronUVc.1141_175insGAp.Jys92ArgfsX10DELc.1376C > Gp.Ser469AsnUVc.1409+42T > AIntronUVc.1409+42T > AIntronUVAmplicon 13c.1558+1G > TSplice defectDEL (splice defect)c.1409+42T > AIntronUVc.1409+42T > AIntronUVAmplicon 14c.1616C > Ap.Arg470XDEL (splice defect)Deletion exon 13Exon deletionDEL (splice defect)c.1409+42T > AIntronUVAmplicon 14c.1616C > Ap.Ala59AspUVAmplicon 15c.1648-19A > GIntronUVAmplicon 16c.1624C > Tp.Gln542XDEL (splice defect)Amplicon 17c.1612-1854delAAGIntronUVAmplicon 16c.1852_1854delAAGp.Pro649Leu582ArgUVAmplicon 17c.16874tr>G			1	
$ c,588+2T>C$ Splice defect DEL (splice defect) Amplicon 8 $c,657A<$	Amplicon 7		Splice defect	DEL (splice defect)
Amplicon 8c.655A > Gp.Ile219ValUV.c700c.676C > Tp.Arg226XDELAmplicon 9c.700+1G > ASplice defectDEL.c700+1G > ASplice defectDEL.c700+3A > TSplice defectDELAmplicon 10c.730C > Tp.Arg265CysUV.c133delCp.Leu272XDEL.c1037A > GO.Gh346ArgUVAmplicon 11c.037A > GO.Gh346ArgUV.c1037A > Gp.Ix9333nsfX34DEL.c1039-8T > A*IntronUV.c1039-15_22del8IntronUV.c117G > Ap.Ser4966AsnUV.c117G > Ap.Ser4960AsnUV.c1376C > Gp.Ser4960AsnUV.c1409+42T > AIntronUV.c158+1G > TSplice defectDEL (splice defect).c1409+42T > AIntronUV.c158+1G > TSplice defectDEL (splice defect).c164C > Tp.Ala539AspUV.c164C > Tp.Ala539AspUV.c1731G > ASplice defectDEL (splice defect).c1731G > ASplice defectDEL (splice defect).c1731+A > GIntronUV.c1754T > GIntronUV.c1754T > GIntronUV.c1754T > GIntronUV.c1754T > GIntronUV.c1754T > GIntronUV.c1852duPTIntronUV.c1852duPTIntronUV.c1852duPTSplice defectD	implicon /		*	
c.676C>T p.Arg226X DEL Amplicon 9 c.702C>A Silent POL c.700+1G>A Splice defect DEL c.700+3A>T Splice defect DEL Amplicon 10 c.793C>T p.Arg265Cys UV c.813delC p.Leu272X DEL Amplicon 11 c.999del p.Gh346Arg UV Amplicon 12 c.1037A>G p.Gh346Arg UV c.1039-15_22del8 Intron UV UV c.1037A>G p.Ser406Asn UV UV c.1376C>G p.Ser406Asn UV UV c.1376C>G p.Ser406Asn UV UV c.1360+32 c.1558+1G>T Splice defect DEL (son deletion) c.1409+42T>A Intron UV UV c.1558+1G>T Splice defect DEL (son deletion) UC c.1558+1G>T Splice defect DEL (son deletion) UL (solice defect) Deletion exon 13 c.1654C>A p.Ala539Asp UV c.1646C>A	Amplicon 8		1	-
Amplicon 9 $c.702$ G>ASilentPOL $c.790+1$ G>ASplice defectDEL $c.790+1$ G>ASplice defectDELAmplicon 10 $c.793$ C>Tp.Arg265CysUV $c.813$ delCp.Luz72XDELAmplicon 11 $c.999$ delp.Lys333Asn(sX34DEL $c.1037$ A>Gp.Gln346ArgUVAmplicon 12 $c.1037$ A>Gp.Gln346ArgUV $c.1037$ A>Gp.Sa32Asn(sX34DEL $c.1039-8T>A^a$ IntronUV $c.1037$ A>Gp.Sa92Arg(sX10DEL $c.137$ ACCp.Sa406AsnUV $c.121$ CG>Ap.Sa406AsnUV $c.121$ CG>Ap.Sa406AsnUV $c.121$ CG>Ap.Sa406AsnUV $c.1368+1G$ SAIntronUV $c.1409+42T>A$ IntronUV $c.158+1G$ SASplice defectDEL (splice defect)Del toin exon 13 $c.1616C>A$ p.Ala539AspUV $c.1616C>A$ p.Ala539AspUV $c.1731+AA>G$ IntronUV $c.1731+AA>G$ IntronUV $c.1731+AA>G$ IntronUV $c.1731+AA>G$ IntronUV $c.1731+AA>G$ IntronUV $c.1731+AA>G$ IntronUV $c.1731+AA>GIntronUVc.1731+AA>GIntronUVc.1731+AA>GIntronUVc.1731+AA>GIntronUVc.1731+AA>GIntronUVc.18720Tp.Glu620XDELc.18740TP.Glo6$			-	
$ C790+1G>A$ Splice defectDELAmplicon 10 $C790+3A>T$ Splice defectDELAmplicon 10 $C793C>T$ $pArg265Cys$ UV $c813delC$ $pLeu272X$ DELAmplicon 11 $c999del$ $pLy333AsnfsX34$ DEL $c1037A>G$ $pGli346Arg$ UVAmplicon 12 $c1039+15_22del8$ IntronUV $c1174_1175insGA$ $pLy392ArgfsX10$ DEL $c127G>A$ $pLy392ArgfsX10$ DEL $c127G>A$ $pLy392ArgfsX10$ DEL $c1409+127DA$ $PLTOPTOPTOPTOPTOPTOPTOPTOPTOPTOPTOPTOPTOPT$	Amplicon 0			
c.790+3A>TSplice defectDELAmplicon 10c.793C>Tp.Arg25CysUVc.813delCp.Leu272XDELAmplicon 11c.999delp.Lys335Anfx343DELc.1037A>Gp.Gln346ArgUVAmplicon 12c.1039-8T>A ⁴ IntronUVc.1039-8T>A ⁴ IntronUVc.1039-52.2del8IntronUVc.1174_1175insGAp.Ser406AsnUVc.1217G>Ap.Ser406AsnUVc.1376C>Gp.Ser406AsnUVc.1408A>Tp.Arg470XDELc.1408A>Tp.Arg470XDELc.158b+1GSTAIntronUVc.158b+1GSAIntronUVc.1405ASTSplice defectDEL (splice defect)Deletion exon 13Exon deletionDELc.1516C>Ap.Ala539AspUVAmplicon 14c.1668-19A>GIntronUVAmplicon 15c.1668-19A>GIntronUVAmplicon 16c.1852_1854delAAGp.Jus618delUVAmplicon 16c.1852_1854delAAGp.Jus618delUVAmplicon 17c.1857dupTp.Glo20XDELAmplicon 17c.1911edGp.Pro649Leufx12DELAmplicon 17c.1957dupTp.Pro649Leufx12DELAmplicon 17c.1957dupTj.Leu55XrgDELAmplicon 17c.1957dupTj.Leu55XrgDELAmplicon 16Lis52_1854delAAGp.Pro649Leufx12DELAmplicon 17c.1957dc>Fj.Leu555XrgDEL <tr< td=""><td>Amplicon 9</td><td></td><td></td><td></td></tr<>	Amplicon 9			
Amplicon 10c.793C>Tp.Arg265CysUVc.813delCp.Lev272XDELAmplicon 11c.999delp.Jys333Asfx534DELc.1037A>Gp.Gln346ArgUVAmplicon 12c.1039-8T>A ^a IntronUVc.1039-15_22del8IntronUVc.117G>Ap.Ser406AsnUVc.1217G>Ap.Ser495XDELc.1408A>Tp.Arg470XDELc.1409+42T>AIntronUVc.1558+14G>AIntronUVc.1616C>Ap.Arg470XDEL (splice defect)Deletion exon 13c.1558+14G>AIntronUVc.1558+14G>AIntronUVc.137G>Gp.Slice defectDEL (splice defect)Deletion exon 13c.1668-19A>GIntronUVc.131G>ASplice defectDEL (splice defect)Amplicon 15c.1668-19A>GIntronUVc.1731G>ASplice defectDEL (splice defect)Amplicon 16c.1652_1554delAAGp.Lys618delUVc.1754T>Gp.Glo52XrgDELAmplicon 16c.1852_1854delAAGp.Lys618delUVc.1857dupTp.Glo620XDELAmplicon 17c.1941delGp.Pro649Leufx12DELc.1959C>Tp.Arg659XDELDELc.1950C>Tp.Arg659XDEL (son deletionDELc.1950C>Tp.Arg659XDEL (son deletionDELc.1950C>Tp.Arg659XDEL (son deletionDEL (son deletionc.1971C>Tp.Arg659X <t< td=""><td></td><td></td><td>-</td><td></td></t<>			-	
$c.813delC$ $p.Lev27X$ DEL Amplicon 11 $c.999del$ $p.Lys333AsnfsX34$ DEL Amplicon 12 $c.1037A > G$ $p.Gln346Arg$ UV Amplicon 12 $c.1039-8T > A^a$ Intron UV $c.1039-15_22del8$ Intron UV $c.1174_c1175insGA$ $p.Lys392ArgfsX10$ DEL $c.1217G > A$ $p.Ser406Asn$ UV $c.1376C > G$ $p.Ser406Asn$ UV $c.1376C > G$ $p.Ser406Asn$ UV $c.1408 + 3T$ $p.Arg470X$ DEL $c.1409+42T > A$ Intron UV $c.1408 + 3T$ $p.Arg470X$ DEL $c.1409+42T > A$ Intron UV $c.1558+1G > T$ $Splice defect$ DEL (splice defect) $Deletion exon 13$ $Exon deletion$ UV $(c.1410_c1558del)^b$ UV $(c.1410_c1558del)^b$ Amplicon 14 $c.1624C > T$ $p.Gln542X$ DEL $(c.1731 + 4A > G$ Intron UV $(c.1731 + 4A >$	A 1' 10		-	
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Deletion exons 17 to 19 Exons deletion DEL (exon deletion) (c.1897_2271del) ^b		c.1975C>T	p.Arg659X	DEL
(c.1897_2271del) ^b		Deletion exons 17 to 19	1 0	DEL (exon deletion)
		(c.1897_2271del) ^b		
Amplicon 18 c.2042dupC p.Met682TvrtsX12 DFI	Amplicon 18	c.2042dupC	p.Met682TyrfsX12	DEL

TABLE 1. Continu	le

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.

^aOnly detected by direct sequencing.

^bLarge 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 $\operatorname{Excel}^{\textcircled{R}}$ (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 $2^{-\Delta\Delta C_P}$ method [Bieche et al., 1998]. The formula used is $N_{ex}=2^{-\Delta\Delta C_P}$, where the $\Delta\Delta C_P$ 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 $2.4 \,\mu$ M in a final volume of $10 \,\mu$ l. 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 SephadexTM 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 homedesigned oligonucleotides on various other cancer predisposition genes), while 1,031 oligonucleotides were specifically homedesigned 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 log₂ 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 +3and -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 \sim 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 wildtype 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/ μ I) andFrom Sample Assays (N = 76).

Amplicons MLH1		qPCR		HRM		
	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.

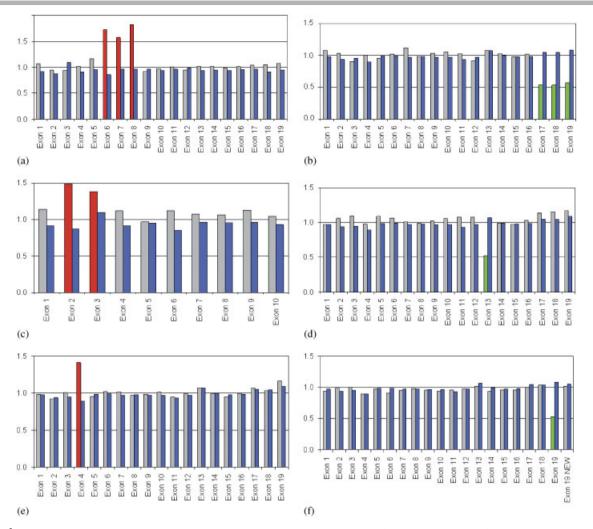


Figure 1. qPCR analysis for each *MLH1* amplicon in normalized N_{ex} to a reference gene (*ALB, ERBB2,* and *MET*). The left gray box is a control and the right blue box represents a patient with a deletion (in green) or a duplication (in red). **A**: Duplication of exons 6 to 8. **B**: Deletion of 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

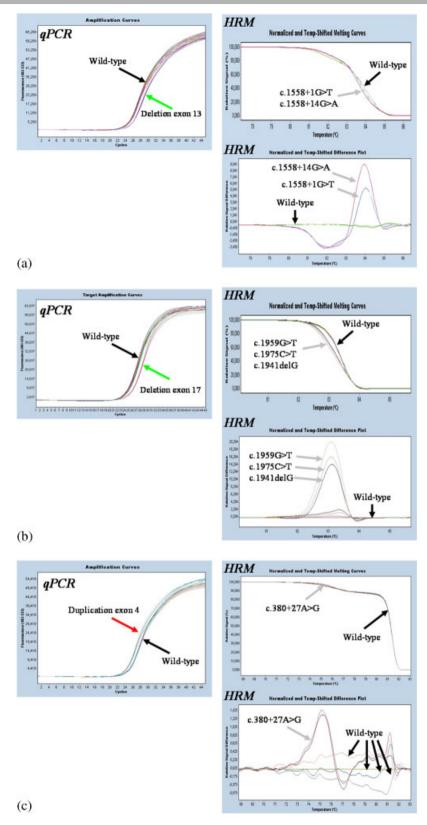


Figure 2. A: Exon 13 of the *MLH1* gene. Left side: qPCR amplification curve with deletion of exon 13. Right side: HRM curves with the mutations c.1558+1G>T and c.1558+14G>A in normalized and temp-shift melting curve and difference plot (duplicate). **B**: Exon 17 of the *MLH1* gene. Left side: qPCR 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.]

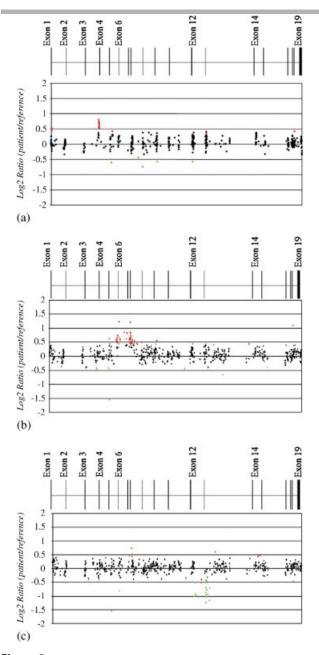


Figure 3. Dedicated zoom-in array-CGH results with the position of exons above and the log_2 ratio of intensity for each oligonucleotide. **A**: Duplication of exon 4 in the *MLH1* gene, precise size (sequenced) 1,665 bp. **B**: Duplication of exons 6 to 8 in the *MLH1* gene with an estimated size between 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 formalinfixed 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, highthroughput 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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