Technical Advance

Detection of BRAF V600E Mutation in Colorectal Cancer

Comparison of Automatic Sequencing and Real-Time Chemistry Methodology

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Mutation V600E of *BRAF*, a kinase-encoding gene from the RAS/RAF/MAPK pathway, in colorectal carcinoma (CRC) suggests a sporadic origin of the disease, providing an exclusion criterion for hereditary nonpolyposis colorectal cancer. Here we describe detection of this mutation by real-time chemistry Taq-Man MGB probes, confirmed by direct DNA sequencing as the gold standard. DNA was extracted from paraffin-embedded tissue from 112 tumors obtained from the EPICOLON study. Seventy-two tumors were CRC with defective DNA mismatch repair (MMR; microsatellite instability and/or loss of protein expression by immunohistochemical analysis), and 40 were proficient MMR controls. *BRAF* mutation was detected in 20/72 (27.8%) CRC with defective MMR and in 3/40 (7.5%) proficient MMR controls ($P = 0.011$). *BRAF* mutation was detected in 19/51 (37.3%) tumors with loss of *MLH1* expression and in none of the tumors with loss of *MSH2* expression (0/13). *BRAF* mutation was not found in cases with germline mutation of *MLH1* (4/112) or *MSH2* (3/112) genes. The sensitivity and specificity of our real-time chemistry were both 100% for detecting the V600E mutation. Because real-

time chemistry methodology has advantages in cost, time, and labor, we consider it a valuable alternative to automatic direct sequencing, particularly for serial measurements. (*J Mol Diagn* 2006, 8:540–543; DOI: 10.2353/jmoldx.2006.060070)

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common hereditary colon cancer syndrome, accounting for 0.9 to 2.5% of all colorectal cancer (CRC).¹ HNPCC is due to a germline mutation of DNA mismatch repair (MMR) genes, mainly *MLH1* or *MSH2*, and cancers occurring in this condition show high-level DNA microsatellite instability (MSI-H). According to the international criteria for HNPCC diagnosis, cancer patients with Amsterdam clinical criteria or Bethesda criteria showing MSI-H should receive genetic counseling and be offered testing for germline mutations of MMR genes.² Furthermore, 5 to 10% of unselected CRC show MSI-H phenotype that is primarily due to hypermethylation of the *MLH1* gene promoter, not germline mutation.³ Thus, it would be desirable to exclude this group from MMR gene testing.

Recently, an oncogenic V600E hotspot mutation in *BRAF*, a kinase-encoding gene from the RAS/RAF/MAPK pathway, has been found in diverse human cancers,⁴ especially in CRC with MMR deficiency.⁵ Interestingly, *BRAF* mutations have not been detected in those cases with a germline mutation in *MLH1*, *MSH2*, or *MSH6*; thus, detection of a positive *BRAF*-V600E mutation in CRC suggests a sporadic origin of the

This work was supported by grant from the Sociedad Española de Oncología Médica (SEOM) and Instituto de Salud Carlos III (CO3/02).

C.A. and S.B. are recipients of a research contract of Spanish Ministry of Health, Instituto de Salud Carlos III (CM04/00035 and 01/3080).

Accepted for publication June 21, 2006.

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disease.⁶ These findings have potential impact for the genetic testing for HNPCC diagnostics and suggest a potential use of *BRAF* as an exclusion criterion for HNPCC.^{7,8}

So far, detection of this mutation has been achieved by restriction fragment length polymorphism analysis,⁹ allele-specific polymerase chain reaction (PCR),¹⁰ single-strand conformation analysis,¹¹ and direct automatic sequencing.⁸ The last method is widely accepted as the standard, but this technique is very time consuming. For this reason, and to perform routine detection of V600E mutation, we assessed the performance of alternative real-time chemistry TaqMan MGB probes in a 7500 sequence detection system designed for allelic discrimination of single nucleotide polymorphisms. The aim of this research was to validate and determine the cost effectiveness of real-time chemistry methodology.

Materials and Methods

Colorectal Cancer Tissue Samples and DNA Extraction

CRC specimens were obtained from researchers participating in the EPICOLON study^{12,13} at Hospital del Mar (M.A., X.B.). The study was approved by the institutional ethics committee, and informed written consent for molecular studies was obtained from all patients. Specimens were previously sequenced for the detection of *BRAF* V600E mutation (Figure 1, A and B). DNA was extracted from paraffin-embedded tissue after deparaffination by the standard procedure involving digestion with proteinase K and phenol-chloroform extraction. Then, extracted DNA was checked and quantified in a Biophotometer (Eppendorf AG, Hamburg, Germany) and stored at -20°C until later use.

Primer-Probe Design

We designed two different sets of primers-probes with Primer Express Software version 2 (Applied Biosystems, Foster City, CA). We generated two different amplicons, one of 176 bp and another of 125 bp. The primers were designed within introns to avoid amplification of a pseudogene located in chromosome X. The chosen primers and probes were subjected to Basic Local Alignment Search Tool (BLAST) N, BLAST X, and BLAST P database searches to find any sequence similarities. The chosen reporter fluorophores for TaqMan MGB probes were VIC (Applied Biosystems) for detecting the wild-type allele and 6-carboxyfluorescein (FAM) for the mutant allele. The two sets of primers-probes were as follows: set 1, *BRAF*-51F (forward) 5'-CTACTGTTTTCTTTACTTACTACACCTCAGA-3', *BRAF*-176R (reverse) 5'-ATCCAGACAACTGTTCAAAGTATG-3', mutant probe 5'-FAM-CTACAGaGAAATCTC-3', and wild-type probe 5'-VIC-AGCTACAGtGAAATC-3'; and set 2, *BRAF*-16F (forward) 5'-GCTTGCTCTGATAGGAAAATGAGATC-3', *BRAF*-176R (reverse) 5'-

Figure 1. Mutation analysis of the *BRAF* gene. An example of sequencing electropherogram with *BRAF* codon V600E heterozygous (A) and wild type (B). C: Amplification plot that displays threshold cycle (Ct) as a function of well position; Ct 0 means no amplification with specific probe, circle is allele T, and square is allele A. D: Allelic discrimination plot that determines the genotype of each tumor. NTC is no template control, Allele X means homozygous allele T, Allele Y means homozygous allele A, and Both means heterozygous alleles T and A.

ATCCAGACAACTGTTCAAAGTATG, mutant probe 5'-FAM-TAGCTACAGaGAAATC-3', and wild-type probe 5'-VIC-CTAGCTACAGtGAAATC-3'. Every possible combination of primers and sets of probes were tested with controls of DNA from the HT29 cell line, which harbors the V600E *BRAF* heterozygotic mutation,⁴ commercial human DNA (Roche Diagnostics GmbH, Mannheim, Germany), and from six previously sequenced DNAs from CRC tissues. We considered the best option the one that was able to discriminate the wild type from heterozygous genotype and that provided the shorter amplicon; thus we chose the primers from set 1 and the probes from set 2.

Real-Time PCR

Once we had chosen our primers and TaqMan MGB probes, real-time PCR was performed with DNA extracted from CRC tissues. Real-time PCR was performed in a final reaction volume of 20 μ l containing 10 μ l of 2 $\times$ TaqMan Universal PCR Master Mix (Applied

Biosystems), 900 nmol/L of each primer, 250 nmol/L of each probe, and 5 μ l of DNA solution. PCR was performed in MicroAmp optical 96-well plates with optical adhesive covers (Applied Biosystems). Amplification and detection were performed with an ABI prism 7500 sequence detection system (Applied Biosystems). The amplification conditions were 2 minutes at 50°C for AmpErase uracil-*N*-glycosylase activity and 10 minutes at 95°C for AmpliTaq Gold activation, followed by 50 cycles of 15 seconds at 92°C for denaturation and 1.5 minutes at 60°C for annealing and extension. After amplification, end point detection of fluorescence was performed at 60°C. The fluorescence data were analyzed with the allelic discrimination software of the ABI Prism 7500 instrument, and amplification plots produced in the PCR reaction were also checked. A time, cost, and labor analysis was also performed to establish the most efficient technique per sample comparing real-time PCR in an ABI Prism 7500 versus automatic sequencing in ABI Prism 310. For real-time PCR versus automatic sequencing, time was established at 4 minutes (6 hours/96 samples) versus 10 hours, cost at 2 € (\$2.60) versus 8 € (\$10.40), and labor at 1.25 minutes (2 hours/96 samples) versus 16 minutes, respectively.

Results and Discussion

We tested DNA from 112 tumors obtained from the EPI-COLON study, a clinical epidemiology survey aimed at establishing the incidence of HNPCC in Spain.^{12,13} Seventy-two tumors were CRC with defective MMR (MSI and/or loss of protein expression in immunohistochemical analysis), and 40 were proficient MMR controls. Mutation analysis of *MLH1/MSH2* was performed in all cases with defective MMR.

BRAF mutation was detected in 20/72 (27.8%) CRC with defective MMR and in 3/40 (7.5%) proficient MMR controls ($P = 0.011$) (Figure 1, C and D). In tumors with loss of *MLH1* expression, *BRAF* mutation was detected in 19/51 (37.3%) tumors and in none of the tumors with loss of *MSH2* expression (0/13). *BRAF* mutation was not found in those cases with germline mutation of the *MLH1* (4/112) or *MSH2* (3/112) genes. These findings are along the same lines as those of other authors^{7,8}.

Considering as gold standard the direct sequencing methodology, the sensitivity and specificity of the real-time PCR procedure were both 100%. Therefore, this real-time PCR method with TaqMan MGB probes is a robust and convenient way to determine the presence of V600E mutation. The use of TaqMan MGB probes gives clear discrimination of genotypes independent of the amount of input DNA.

In conclusion, real-time PCR is a highly sensitive and specific methodology for detecting V600E mutation. Because real-time chemistry methodology has advantages in cost, time, and labor, we consider that it may be an alternative to automatic direct sequencing, particularly for serial measurements. The combination of TaqMan

MGB probes with universal assay conditions allows an efficient workflow when the number of different single-nucleotide polymorphisms that are detected in the clinical laboratory increases.

Acknowledgments

We thank Dolores Durá, Juan Pulido, and Estefania Rojas for technical assistance.

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