High-Resolution Melting Analysis for Rapid Detection of *KRAS, BRAF,* and *PIK3CA* Gene Mutations in Colorectal Cancer

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Abstract

High-resolution melting analysis (HRMA) provides a valid approach to efficiently detect DNA genetic and somatic mutations. In this study, HRMA was used for the screening of 116 colorectal cancers (CRCs) to detect hot-spot mutations in the KRAS *and* BRAF *oncogenes. Mutational hot spots on the* PIK3CA *gene, exons 9 and 20, were also screened. Direct sequencing was used to confirm and characterize HRMA results. HRMA revealed abnormal melting profiles in 65 CRCs (56.0%), 16 of them harboring mutations in 2 different genes simultaneously. The frequency of mutations was 17.2% for* PIK3CA *(11.2% in exon 9 and 6.0% in exon 20), 43.1% for* KRAS *exon 2, and 9.5% in exon 15 of the* BRAF *gene. We found a significant association between* PIK3CA *and* KRAS *mutations (*P *= .008), whereas* KRAS *and* BRAF *mutations were mutually exclusive (*P *= .001). This report describes a novel approach for the detection of* PIK3CA *somatic mutations by HRMA.*

The request for rapid and reliable mutation screening in human cancers is rapidly increasing for the definition of clinical samples and orienting targeted therapies. High-resolution melting analysis (HRMA) was recently proposed as a very sensitive scanning method that allows rapid detection of DNA sequence variations without cumbersome post–polymerase chain reaction (PCR) methods.[1-3] Mutation scanning with HRMA is based on the dissociation behavior of DNA when exposed to an increasing temperature. Signal modification is generated from the transition from a double to single strand in the presence of fluorescent dyes actively intercalating double-stranded DNA.[4] The HRMA melting profile gives a specific sequence-related pattern allowing discrimination between wild-type sequences and homozygote-heterozygote variants.[5] Applications of HRMA have been described for the identification of germline and somatic mutations.[6-11] Owing to its high sensitivity, HRMA seems to represent a more sensitive approach to detect a minimal fraction of mutated cells in tumoral tissue that, in many cases, is not achievable by direct sequencing.

In the present study, we used HRMA for the rapid and sensitive detection of somatic mutations in hot-spot regions of 3 oncogenes (*KRAS*, *BRAF,* and *PIK3CA*) that are frequently mutated in colorectal cancer (CRC).

RAS proteins belong to an effector family that regulates intracellular signaling pathways involved in proliferation, survival, and differentiation.[12] Cancer-associated mutations responsible for gain-of-function of *RAS* genes have been described in non–small cell lung cancer,[13-15] in the biliary tract,[12] and in pancreas.[12] Missense *RAS* mutations essentially cause substitutions at codons 12, 13, and 61, transforming the intrinsic GTPase activity of the protein in the constitutively

active conformation. In CRC, *KRAS* mutations occur in 30% to 51% of cases[12,16-18] and are inversely associated with *BRAF* mutations, suggesting that their involvement in cancer progression can independently induce similar cellular effects through the same pathway.[16] Recently, HRMA was proposed for use in screening for *KRAS* mutations in human lung cancer.[15]

BRAF is a cytosolic protein kinase and is activated by membrane-bound RAS. Mutated BRAF activates a signaling cascade involving proteins in the mitogen-activated protein kinase system, resulting in cell proliferation.[19] *BRAF* mutations occur in about 10% of CRCs, with the most common mutation being the T to A transversion at nucleotide 1796, causing V600E. This mutation predisposes to inhibition of apoptosis, increases invasiveness,[20] and occurs earlier during colorectal carcinogenesis.[21] The *BRAF* V600E mutation is mutually exclusive with *KRAS* mutations and is significantly associated with microsatellite instability, resulting from aberrant methylation of the MLH1 promoter.[22] HRMA has been previously proposed for use in screening for *BRAF* mutations in human melanoma.[23]

We also performed HRMA to identify hot-spot gain-of-function mutations in exons 9 and 20 in *PIK3CA*, the catalytic subunit of phosphatidylinositol 3-kinase (PI3K). PI3Ks are heterodimeric kinases involved in the control of cellular growth, transformation, adhesion, and apoptosis.[24-26] Somatic mutations of *PIK3CA* were described in CRCs with a frequency ranging from 13.6% to 32%.[24,25,27,28] In CRCs, 3 mutational hot spots were described (E542K, E545K, and H1047R), covering about 80% of all *PIK3CA* mutations.[24] The first two are located in the helical domain, and the third is in the C-terminal region of the protein.[26,28] These gain-of-function mutations confer strong oncogenicity as shown by in vivo[29] and in vitro assays.[30] Recently, a significant correlation between *PIK3CA* mutations and patient survival was found in CRC,[28] suggesting that screening for *PIK3CA* mutations could be useful in the selection of patients for adjuvant therapy.

Materials and Methods

Samples

Tissue samples were obtained from 116 consecutive patients with sporadic CRC (60 men and 56 women; age range, 43-89 years; mean, 65.8 years). Informed consent was obtained from all patients. Samples were snap frozen and stored in liquid nitrogen until analysis. Tumor histologic type and grade of differentiation were defined according to the World Health Organization criteria[31] and staged according to the American Joint Committee on Cancer TNM staging system.[32] DNA extraction was performed by using BIOROBOT EZ1 (QIAGEN, Milan, Italy) and the EZ1 DNA Tissue Kit (QIAGEN) according to the manufacturer's protocol.

Cell Lines

Positive control samples were obtained from DNA of cell lines harboring mutations in the target genes, as reported by Ikediobi et al.[33] SW620 and HCT116 were used as reference for *KRAS* codon 12 (G12V, homozygous) and 13 (G13D, heterozygous) mutations, respectively; for exons 9 and 20 of *PIK3CA,* we used MCF-7 (E545K, heterozygous) and HCT116 (H1047R, heterozygous), respectively. SK-MEL-28 (V600E, homozygous) and MDA-MB-231 (G464V, heterozygous) were the references for exons 11 and 15 of the *BRAF* gene. DNA was extracted from cell lines by using the QIAamp DNA Mini Kit (QIAGEN).

High-Resolution Melting Analysis

For HRMA, 100 ng of DNA was amplified in a final volume of 10 μL by using the following: 1.25 μL of PCR Buffer (Applied Biosystems, Monza, Italy), 1.5 mmol/L of magnesium chloride (Applied Biosystems), 300 nmol/L of each primer, 1.5 μmol/L of SYTO9 Dye (Invitrogen, Carlsbad, CA), and 1.25 U of *Taq* Gold Polymerase (Applied Biosystems). PCR was submitted in a 9700 GeneAmp PCR system (Applied Biosystems) thermal cycler to an initial denaturation at 95°C for 10 minutes followed by 35 cycles of 1 minute at 95°C, 1 minute at 60°C, and 1 minute at 72°C and a final extension at 72°C for 15 minutes. For the HRMA melting profile, performed on the RotorGene 6000 (Corbett Research, Sydney, Australia), samples were denatured with an initial hold of 5 minutes at 95°C and 1 minute at 40°C and a melting profile from 75°C to 85°C for *KRAS* and *BRAF*; 77°C to 87°C for *PIK3CA* exon 20 and 70°C to 85°C for *PIK3CA* exon 9, with a ramping degree of 0.05°C. Primers for HRMA analysis were selected using Primer3 software[34] and were as follows: *KRAS,* 5′-GCCTGCTGAAAATGACTGAA-3′ (forward), 5′-AGAATGGTCCTGCACCAGTAA-3′ (reverse); *PIK3CA* exon 9, 5′-CTAGCTAGAGACAATGAATTAAGGGAAA-3′ (forward), 5′-CATTTTAGCACTTACCTGTGACTCCA-3′ (reverse); *PIK3CA* exon 20, 5′-TGAGCAAGAGGCTTTGGAGT-3′ (forward), 5′-TCATTTTCTCAGTTATCTTTTCAGTTCAAT-3′ (reverse). Screening for *BRAF* gene exons 11 and 15 was performed as previously reported.[23]

DNA Sequencing

To confirm HRMA results, sequencing analysis was also performed in all samples. After HRMA, samples were purified with a PCR Purification Kit (QIAGEN) and submitted to cycle

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sequencing with 2 μL of BigDye Terminator Ready Reaction Mix (Applied Biosystems) and the same primers used in HRMA but 0.8 μmol/L in a final volume of 10 μL. After purification with a DyeEx 2.0 Spin Kit (QIAGEN), samples were analyzed with the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

Statistical analysis was carried out using the SPSS software package (SPSS, Chicago, IL). Statistical differences between groups were assessed by using the χ^2 test, considering the *P* value as obtained by using the Fisher exact test. Differences with a *P* value of less than .05 were considered statistically significant.

Results

Reliability and Sensitivity of HRMA

Sensitivity of the melting profile in discriminating different percentages of mutated alleles was initially evaluated by using serial dilutions of mutated DNA, derived from cultured cell lines, variably mixed with wild-type DNA, obtained from wild-type cell lines. We tested 100%, 75%, 50%, 25%, 15%, 10%, and 5% (mutated/wild type) dilutions of SW620 (mutated in codon 12 of *KRAS*) and SK-MEL-28 cell lines (mutated in exon 15 of *BRAF*), both carrying the respective mutation in homozygosis. Our results confirmed that the technique can detect at least 5% of mutated alleles (data not shown). For MCF-7 (*PIK3CA* in exon 9) and HCT-116 (*PIK3CA* in exon 20) that contain mutations in heterozygosis, samples were diluted at 50%, 25%, 12%, 6%, and 3% **Figure 1**. We were able to identify the presence of an abnormal profile of HRMA in all dilutions, allowing the clear identification of mutated alleles (Figure 1).

HRMA and Sequencing Analysis

Examples of HRMA and melting profiles obtained from CRCs carrying mutations of *KRAS* and *BRAF* are shown in **Figure 2**. HRMA profiles of exons 9 and 20 of *PIK3CA*, with direct comparison of sequencing results, are shown in **Figure 3** and **Figure 4**, respectively. A clear definition of the presence of somatic mutations in a sample was achievable by the comparison of the profile with wild-type DNA. Sequencing analysis was performed in all samples using the same PCR products after melting analysis. In all cases, dideoxy sequencing confirmed the results of HRMA.

We found at least 1 mutation in 65 (56.0%) of 116 cases, with 16 of them harboring 2 mutations simultaneously. Nine variants in the *KRAS* gene were detected with a 43.1% global frequency **Table 1**. Among them, the G12D substitution was the most prevalent (42%). In our cases we also detected uncommon *KRAS* mutations: 1 case with Q22K mutation and 1 case with L19F, recently described as an activating mutation.[35] In 1 case, HRMA and sequencing analysis identified an

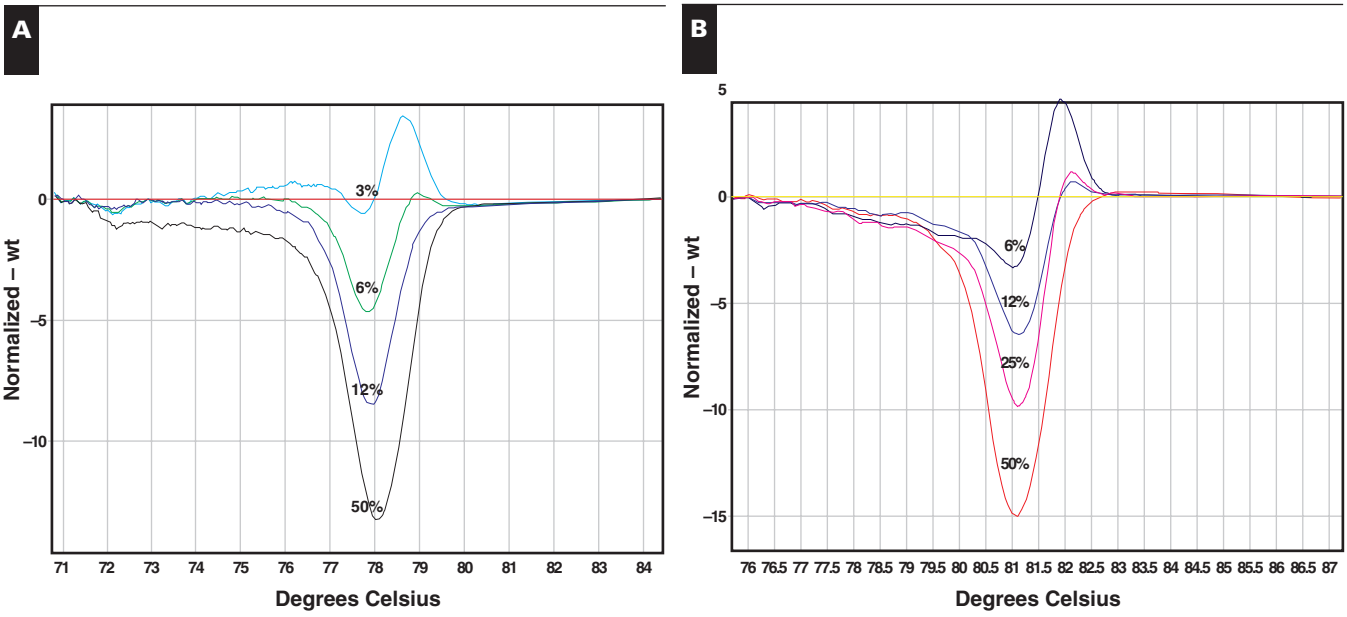

Figure 1 Difference plots generated with serial dilutions of DNA from mutated cell lines with wild-type (wt) DNA to assess high-resolution melting analysis sensitivities. **A**, *PIK3CA* exon 9 mutation detected using MCF-7 DNA. **B**, *PIK3CA* exon 20 mutation detected by HCT116. Percentages of relative amount of mutated allele are also reported.

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additional variant: an in-frame c.30_31insGGA insertion (Figure 2A) resulting in a glycine insertion (p.10_11insG) that has not been reported before in CRC. This mutation was previously described only in 1 case of childhood myeloid leukemia and was tested for its activating features.[36] In the *BRAF* gene, no mutation was found in exon 11, whereas the final frequency of the V600E mutation in exon 15 was 9.5%.

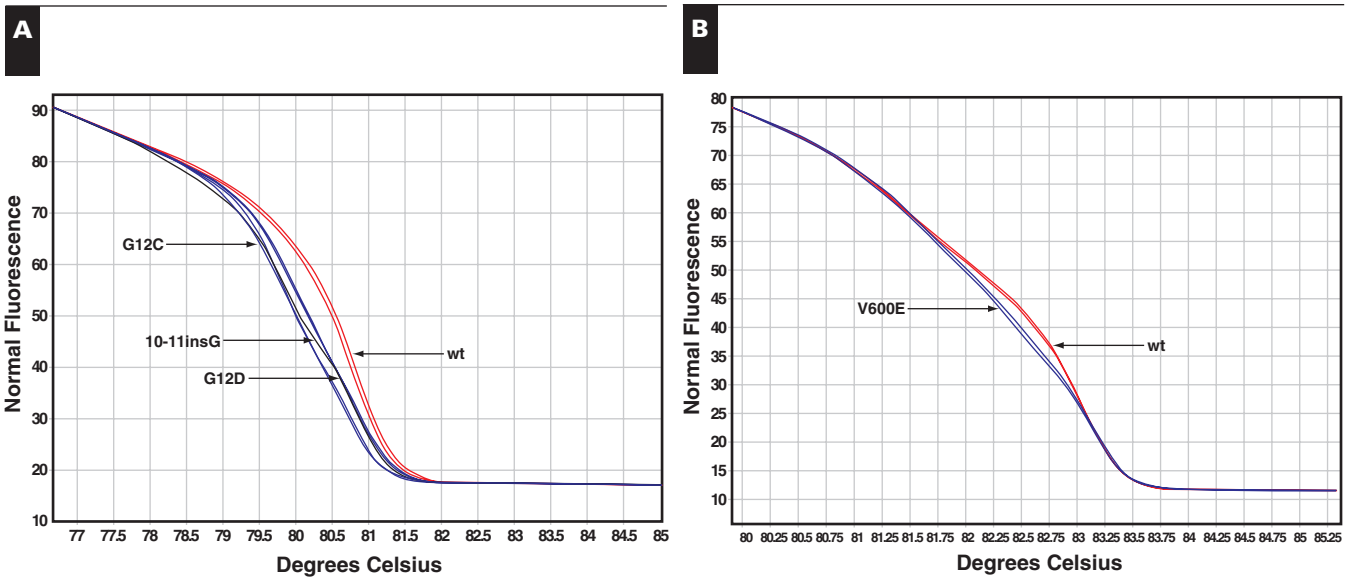

Figure 2 High-resolution melting analysis of *KRAS* (**A**) and *BRAF* exon 15 (**B**). Arrows indicate wild-type (wt) control and colorectal cancer samples with somatic mutations.

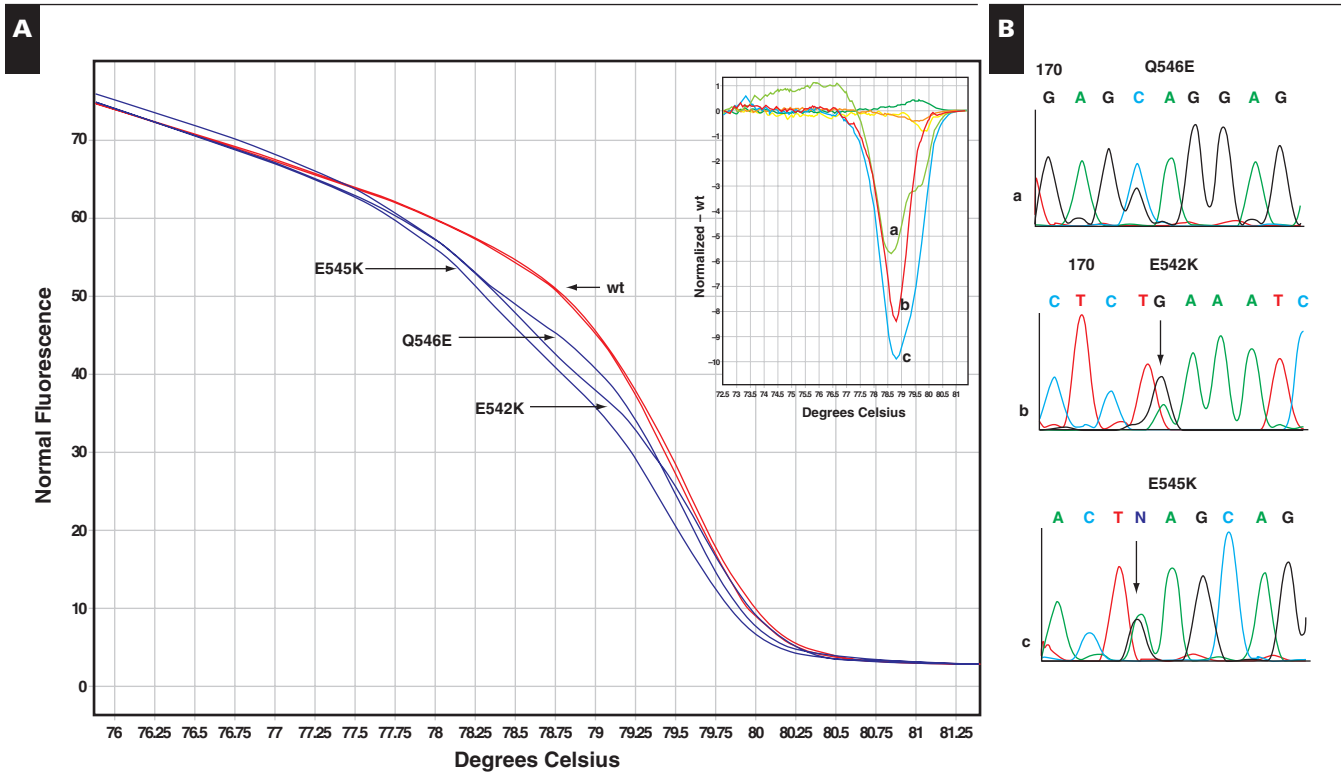

Figure 3 High-resolution melting analysis of *PIK3CA* exon 9. **A**, Melting profiles with arrows that indicate wild-type (wt) control and colorectal cancer samples with somatic mutations. Inset, Difference plots of mutated samples normalized against wt DNA, **B**, Electropherograms of sequencing analysis in the same samples.

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For the *PIK3CA* gene, among the 7 variants we identified, E545K, E542K, and H1047R were confirmed as the most frequent mutations for exons 9 and 20 (15/20 [75%]). The final frequency of hot-spot mutations involving *PIK3CA* was 20 (17.3%) of 116 (Table 1).

The analysis of our results confirmed that *KRAS* and *BRAF* mutations are mutually exclusive ($P = .001$) because none of the 50 CRCs with *KRAS* mutations had a concomitant mutation in *BRAF*. On the contrary, *PIK3CA* and *KRAS* correlated significantly (P = .008) because 14 (70%) of 20 CRCs carrying 1 mutation in the *PIK3CA* gene had also 1 mutation in the *KRAS* gene. In particular, all *PIK3CA* mutants in exon 20 (n = 7) are concomitant with *KRAS* mutations, whereas only 7 (54%) of 13 of exon 9 mutants are associated with *KRAS* mutations. In 2 (10%) of 20 CRCs with *PIK3CA* exon 9 mutations, a *BRAF* mutation was also present.

Table 1
Number of Variants Detected by High-Resolution Melting Analysis and Defined by Direct Sequencing for the *KRAS*, *BRAF*, and *PIK3CA* Genes

Gene/Nucleotide Change	Protein Mutation	No. of Samples	Relative %	Total %*
KRAS				
c.35G>A	G12D	21	42	18.1
c.35G>T	G12V	8	16	6.9
c.38G>A	G13D	7	14	6.0
c.34G>T	G12C	6	12	5.2
c.35G>C	G12A	4	8	3.4
c.34G>C	G12R	1	2	0.9
c.57G>T	L19F	1	2	0.9
c.64C>A	Q22K	1	2	0.9
c.30_31insGGA	p.10_11insG	1	2	0.9
Total		50	100	43.1
BRAF				
c.1799T>A	V600E	11	100	9.5
PIK3CA				
Exon 9				
c.1633G>A	E545K	6	30	5.2
c.1624G>A	E542K	4	20	3.4
c.1637A>T	Q546L	2	10	1.7
c.1636C>G	Q546E	1	5	0.9
Exon 20				
c.3140A>G	H1047R	5	25	4.3
c.3129G>T	M1043I	1	5	0.9
c.3127A>G	M1043V	1	5	0.9
Total		20	100	17.3

* Of 116 cases.

Discussion

The main purpose of this study was to develop a sensitive test that allows rapid identification of hot-spot mutations in *PIK3CA*, *KRAS,* and *BRAF* oncogenes in CRC. In recent years, the research and clinical management of CRC have

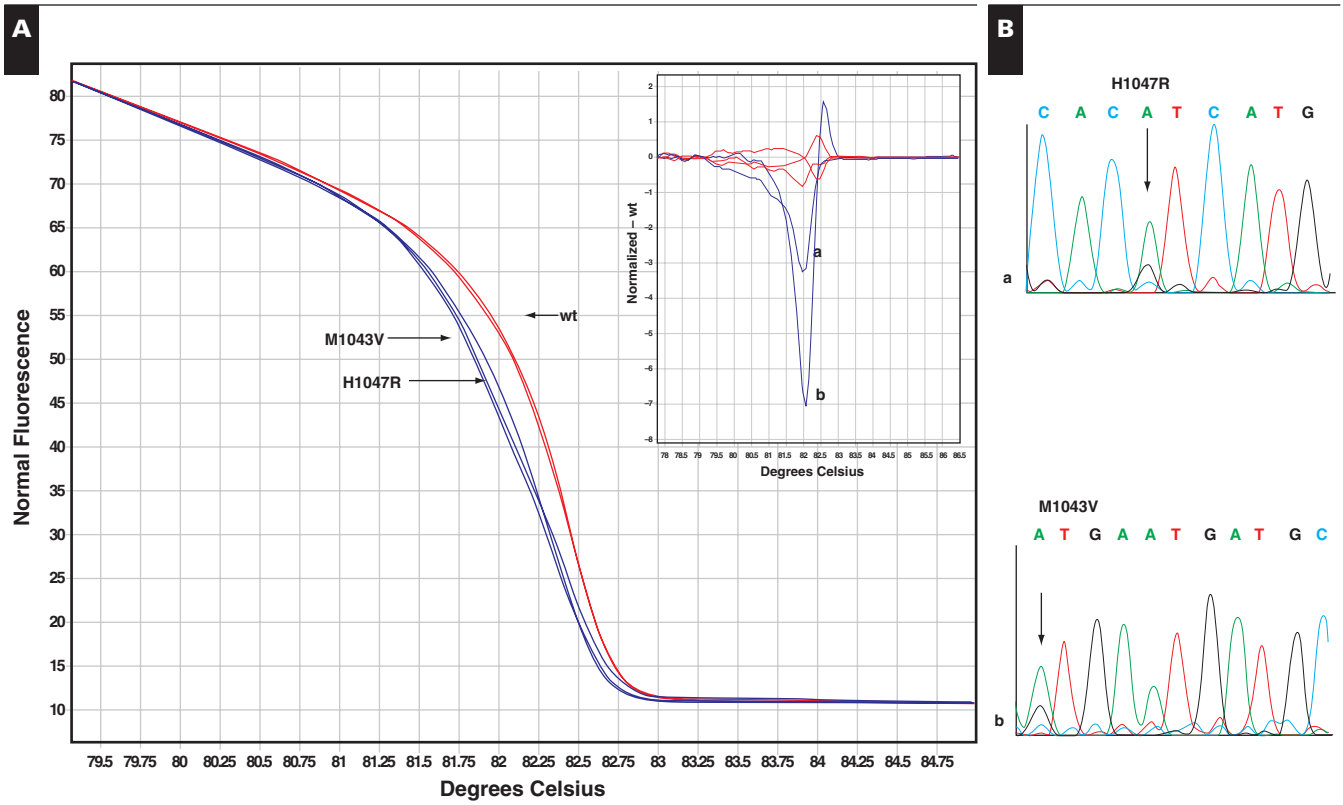

Figure 4 High-resolution melting analysis of *PIK3CA* exon 20. **A**, Melting profiles with arrows that indicate wild-type (wt) control and colorectal cancer samples with somatic mutations. Inset, Difference plots of mutated samples normalized against wt DNA. **B**, Electropherograms of sequencing analysis in the same samples.

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changed and been revised on the basis of genetic features that characterize this tumor.[17,37] The involvement of independent pathways in the carcinogenesis of CRC is supported by the existence of great variability in the pattern of somatic mutations. Further investigations should be performed to clarify the clinical role of single or multiple somatic mutations, and it is then reasonable to insist on the development of achievable genetic tests to facilitate patient stratification for targeted therapeutic approaches. Therefore, rapid detection of multiple mutations with high accuracy in a series of known oncogenes will be more often requested in future clinical management.

In a mutational study on 1,000 human tumor samples, Thomas et al[38] confirmed that changes affecting a small number of codons often account for the majority of somatic mutations. In the same adapted high-throughput genotyping, the authors also demonstrated that *PIK3CA*, *KRAS,* and *BRAF* genes could be regarded as colorectal-specific somatic mutations.[38] Even if it is widely accepted that some oncogene mutations occur in a mutually exclusive or inclusive manner, it remains to be clarified when and where they can really interfere with common pathways. Controversial data are reported on the correlation between *PIK3CA* and *KRAS* mutations, and it is still unclear whether mutations that affect both genes could effectively influence the phenotypic features of the tumor. In a recent study, *KRAS* and *PIK3CA* mutations were reported to be not correlated in CRC,[28] whereas, according to the study by Thomas et al,[38] about 30% of *PIK3CA* somatic mutations couple with another oncogene mutation, mainly represented by the *KRAS* gene. Our data indicate that in 14 (70%) of 20 CRCs with a *PIK3CA* mutation, there was a concomitant mutation in *KRAS* ($P = .008$). In particular, 100% of cases with *PIK3CA* mutations in exon 20 were simultaneously mutated in *KRAS*, whereas only 54% of CRCs carrying an exon 9 mutant shared a mutation with *KRAS*. Finally, our data confirmed that mutations in *BRAF* were mutually exclusive of *KRAS* mutations.

HRMA provides a reliable and cost-limited approach to perform genetic screenings in tumor samples. Our experiments on reconstituted samples, obtained by serial dilutions of mutated cancer cell lines and normal DNA, indicated the possibility of identifying at least 5% of mutated alleles in a background of wild-type DNA. This theoretical sensitivity seems well suited for detecting even a limited percentage of mutated alleles in a heterogeneous sample, as obtained from CRC random tissue biopsies. The whole procedure seems very rapid, eliminating the need for cumbersome and technically demanding prescreening tests. In addition, HRMA was demonstrated to be useful in the identification of rare *KRAS* mutations. We identified 1 case of an in-frame c.30_31insGGA insertion that was responsible for the insertion of an extra glycine. This insertion, even previously demonstrated in 1 infant with leukemia, has not been reported before in colorectal cancer. This mutation was reported to induce cell transformation in vitro and activation of the Ras-mitogen-activated protein kinase signaling pathway.[36] This observation is in accordance with the finding that synthetic mutants with insertion of extra amino acids in the phosphate-binding P-loop of KRAS (codons 10-17) can induce a reduction of affinity for guanosine 5′-diphosphate, leading to a preference for guanosine 5′-triphosphate binding and promoting outgrowth comparable to single point *KRAS* mutants.[39]

Our results seem to point out the importance of new rapid and sensitive procedures for accurate screening for somatic mutations to detect common and rare oncogene mutants in CRCs. HRMA seems to be a relevant tool for this type of study.

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