The Use of COLD-PCR and High-Resolution Melting Analysis Improves the Limit of Detection of *KRAS* and *BRAF* Mutations in Colorectal Cancer

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Fast and reliable tests to detect mutations in human cancers are required to better define clinical samples and orient targeted therapies. *KRAS* mutations occur in 30–50% of colorectal cancers (CRCs) and represent a marker of clinical resistance to cetuximab therapy. In addition, the *BRAF* V600E is mutated in about 10% of CRCs, and the development of a specific inhibitor of mutant BRAF kinase has prompted a growing interest in *BRAF* [V600E] detection. Traditional methods, such as PCR and direct sequencing, do not detect low-level mutations in cancer, resulting in false negative diagnoses. In this study, we designed a protocol to detect mutations of *KRAS* and *BRAF*[V600E] in 117 sporadic CRCs based on coamplification at lower denaturation temperature PCR (COLD-PCR) and high-resolution melting (HRM). Using traditional PCR and direct sequencing, we found *KRAS* mutations in 47 (40%) patients and *BRAF*[V600E] in 10 (8.5%). The use of COLD-PCR in apparently wild-type samples allowed us to identify 15 newly mutated CRCs (10 for *KRAS* and 5 for *BRAF* [V600E]), raising the percentage of mutated CRCs to 48.7% for *KRAS* and to 12.8% for *BRAF* [V600E]. Therefore, COLD-PCR combined with HRM permits the correct identification of less represented mutations in CRC and better selection of patients eligible for targeted therapies, without requiring expensive and time-consuming procedures. (*J Mol Diagn 2010, 12:705–711; DOI: 10.2353/jmoldx.2010.100018*)

Cancer biomarkers play multiple roles in oncology. They can have prognostic functions by providing information on outcome and patient tractability. They can also play a predictive role by assessing the probability that patients will benefit from specific treatments. Finally, some cancer biomarkers possess pharmacodynamic criteria, measuring drug effects and orienting dose selection.[1]

Cancer genotyping is now often requested to tailor personalized therapies in the treatment of common cancers, like colorectal[2,3] breast,[4] lung,[5] prostate,[6] and head and neck.[7] In colorectal cancer (CRC) the search for genetic markers for therapy response could help reduce the toxicity from therapy in patients who would not benefit from treatment.[8] Selecting patients who are likely to take advantage of targeted treatments will ultimately result in better patient outcome and reduced costs.[2]

Somatic mutations in *KRAS* are considered a predictive marker of response to therapy in CRC, due to their association with clinical resistance to cetuximab and panitumumab, chimeric monoclonal antibodies acting as inhibitors of the epidermal growth factor receptor.[9–12] These drugs improve a variety of clinically important outcomes in CRC patients who have the wild-type *KRAS* gene, whereas no response is observed in CRC patients with *KRAS* mutations.[2,8,13,14] *KRAS* activating mutations have been reported in 30–54% of metastatic CRC patients,[13–15] resulting in EGFR-independent activation of the mitogen-activated protein kinase pathway.

BRAF is mutated in about 10% of CRC patients, and the T>A transversion at nucleotide 1796, causing V600E, accounts for the most frequent of all BRAF variants. The high incidence of *BRAF*[V600E] mutation in cancer suggests that *BRAF* may also be an attractive therapeutic target[16] and negative predictors of cetuximab therapy.[17] The recent development of a specific inhibitor of the mutant isoform of BRAF kinase, currently in clinical trials (PLX4032),[18] has increased the value of *BRAF*[V600E] identification in cancer patients who could benefit from this therapy.

The prognostic role of *KRAS* and *BRAF* in CRC has also been well investigated.[19] *KRAS* mutations generally confer a worse prognosis in CRC patients, even if convincing evidence of the independent prognostic role of *KRAS* mutations is still lacking.[2] Although the association of *BRAF* with prognostic parameters is still controversial, its pattern of mutations that mutually exclude *KRAS* seems to confirm its role in orienting patient therapy.[20,21]

Due to the cellular heterogeneity of solid cancers, the primary technical challenge encountered in the detection of somatic variants is the cellular heterogeneity in tumor biopsies. Somatic mutations can be present in low amounts within an elevated background of wild-type sequences, and more sensitive assays are therefore needed than those used for germline variants. There are many new approaches for improving sensitivity and efficiency detection of mutant alleles, such as high-resolution melting analysis,[22–25] pyrosequencing,[26,27] real-time PCR.[28,29] Finally the use of Locked Nucleic Acid (LNA) and Peptide Nucleic Acid (PNA)[30] was also proposed.

Although having higher sensitivity in variant detection, these approaches do not have an advantage for mutant alleles during the amplification protocol, which may be the only time when enrichment of variant alleles would be requested in samples with a very low percentage of mutated DNA.

Coamplification at lower denaturation temperature PCR (COLD-PCR) is a recently introduced PCR method that allows preferential amplification of minority alleles from a mixture of wild-type and mutant sequences.[31] The principle of this approach derived from the direct relationship between a given DNA sequence and the relative critical denaturation temperature (T_c). Sequence mismatch (heteroduplex), caused by point mutations, are responsible of an earlier denaturation step, thus by using a lower denaturation temperature during PCR, a selective amplification of mutant alleles will be performed.[28,29] On this basis, the COLD-PCR application first requires definition of the optimal dissociation temperature (T_d), commonly defined as critical temperature (T_c), at which the enrichment of minority alleles during PCR amplification is maximized.

COLD-PCR has been already tested in the detection of *KRAS*,[31,32] *TP53*,[31,33] *EGFR*,[31] and *GNAS1*[34] mutations, and can identify mutated samples not otherwise identified by conventional PCR and cycle sequencing.

Mutation scanning with high-resolution melting analysis, HRMA, is based on the dissociation behavior of DNA when exposed to increasing temperatures. The signal modification is generated from the transition from double-to-single strand in the presence of fluorescent dyes actively intercalating double-stranded DNA.[35] The HRM melting profile gives a specific sequence-related pattern that differentiates wild-type sequences from homozygote or heterozygote variants.[36] As recently reported, combination of COLD-PCR and HRMA could further improves the mutation-scanning capabilities of HRM.[37]

In the present study we describe a COLD-PCR and HRM approach for sensitive detection of *KRAS* and *BRAF* mutations in 117 CRC patients.

Materials and Methods

Tumor Samples

Tissue samples were obtained from 117 consecutive patients with sporadic CRC (60 men and 57 women; mean age 67.5 years, range 48–89). A fragment of cancer tissue was snap-frozen in liquid nitrogen, while the re-

maining was processed for routine histological examination. The study was approved by the local ethical committee and an informed consent was obtained from each patient. Cancer histology and grading were defined using the World Health Organization criteria.[38] CRCs were staged according to the American Joint Committee on Cancer TNM staging system.[39] DNA extraction from snap-frozen tissues was performed by using EZ1 BIOROBOT and the EZ1 DNA Tissue Kit (Qiagen Inc., Germany) following to the manufacturer's protocol.

Cell Lines

DNA from SK-Mel-28 cell line, harboring the homozygous *BRAF*[V600E] mutation, and CCRF-CEM heterozygous for G12D mutation in *KRAS* was used as control. Mutated DNAs were diluted with MCF-7 wild-type DNA to obtain a variable percentage of mutated alleles. Even if MCF-7 cells are known to be an aneuploid cell line, we did not consider this aspect in the context of the dilution experiments a major problem. DNA extraction was performed by using QIAamp DNA Mini Kit (Qiagen, Milan, Italy).

Optimization of COLD-PCR for KRAS

PCR and COLD-PCR for *KRAS* codons 12 and 13 were initially performed using the same primer set already described by Zuo et al.[31] During the reevaluation of the critical temperature of COLD-PCR with this primer set, we observed that the decrease in denaturation temperature (from 82.5 to 81°C) generated a double product of amplification. The first corresponded to the expected *KRAS* gene sequence, while the second was identified as the *KRAS* pseudogene (KRAS1P, GenBank NC_000006) located on chromosome 6 (data shown in Supplemental Figure 1, available at *http://jmd.amjpathol.org*). The two sequences have the same size (98 bp) but have a four-bases difference in the amplified region, which reduces the melting temperature (T_m) for pseudogene sequence. This fact favors its preferential amplification in comparison with the *KRAS* gene at a lower denaturation temperature (T_d). At 81.5°C T_d only pseudogene sequence was amplified. This limitation was incompatible with the definition of a correct critical temperature (T_c) for minority allele enrichment. For this reason we designed a new set of primers to amplify a fragment of 155 bp and containing a mismatch at 3′ to prevent pseudogene amplification: forward 5′-GTCACATTTTCATTATTTTTATTATAAGG-3′ and reverse 5′-TTTACCTCTATTGTTGGATCATATTC-3′.

Testing a series of T_d (from 83°C to 81°C) in COLD-PCR experiments, we found that 82.5°C was the T_c for fast COLD and 82°C for full COLD formats. These temperatures provided the best mutant-sequence enrichment, with efficient amplification.

PCR and COLD-PCR for KRAS

Reactions for conventional PCR and COLD-PCR were performed in a RotorGene 6000 (Corbett Research Pty Ltd, Sidney, Australia) using 20 ng of genomic DNA in a

total volume of 30 μl containing a final concentration of 1× PCR Buffer II (10 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl) (Applied Biosystems, Milan, Italy), 2.5 mmol/L MgCl Solution, 0.2 mmol/L each dNTP, 0.5 μmol/L each primer, 0.5 μmol/L Syto9 (Invitrogen Corp., Carlsbad, CA) and 1.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems). Amplification by conventional PCR was performed with an initial hold at 95°C for 5 minutes, 45 cycles at 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 20 minutes.

COLD-PCR was performed using two alternative protocols: the 'fast' protocol, to detect Tm-reducing variants, and the 'full' protocol, suitable for revealing Tm-retaining and Tm-increasing alleles, as previously indicated.[30] Because fast COLD-PCR guarantees higher enrichment of mutant alleles with G>T or G>A substitutions, which are the most frequent mutations in the *KRAS* gene, this approach was initially used to screen all samples. Then, the full protocol was used to further increase sensitivity, allowing other variant detection (ie, G>C and insertion) despite less enrichment of mutant alleles. The fast COLD-PCR protocol included an initial step at 95°C for 5 minutes followed by 20 PCR cycles at 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by 35 cycles at specific Tc: dissociation at 82.5°C for 3 seconds, 58°C for 30 seconds, 72° for 30 seconds, and a final extension at 72°C for 20 minutes. Full COLD-PCR cycles had an initial step at 95° for 5 minutes. The 15 cycles of PCR were performed at 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, followed by

40 cycles at specific steps: 95° for 15 seconds, 70°C for 7 minutes, 82°C for 3 seconds, 58°C for 30 seconds, 72° for 30 seconds, with a final extension at 72°C for 20 minutes.

PCR and COLD-PCR for BRAF

Primers selected for conventional and COLD-PCR for *BRAF* exon 15 were forward 5′-ACAGAATTATAGAAAT-TAGATCTCTTACC-3′ and reverse 5′-GACAACTGT-TCAAACTGATGG-3′, which amplify a 200-bp fragment. Reactions were performed using 20 ng DNA in the QuantiTect Probe PCR Master Mix (Qiagen) with 300 nmol/L of each primer and 1.5 μmol/L of Syto9 (Invitrogen) in a final volume of 20 μl. PCR was performed as follows: an initial hold at 95°C for 15 minutes, 40 cycles to 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 15 minutes. Because this type of mutation introduces a T>A transversion, a full COLD-PCR was directly used. Preliminarily, we tested a range of Td in COLD-PCR experiments (from 80°C to 78°C) and we found that 79.5°C was the best Tc for COLD. These temperatures provided the best mutant-sequence enrichment, with efficient amplification.

The full COLD-PCR protocol was as follows: 95°C for 15 minutes; 10 cycles of conventional PCR: 95°C for 15 seconds; 62°C for 30 seconds; 72°C for 1 minute; then 35 cycles of COLD-PCR performed at 95°C for 15 seconds, 70°C for 7 minutes, 79.5°C for 3 seconds, 60°C for 30
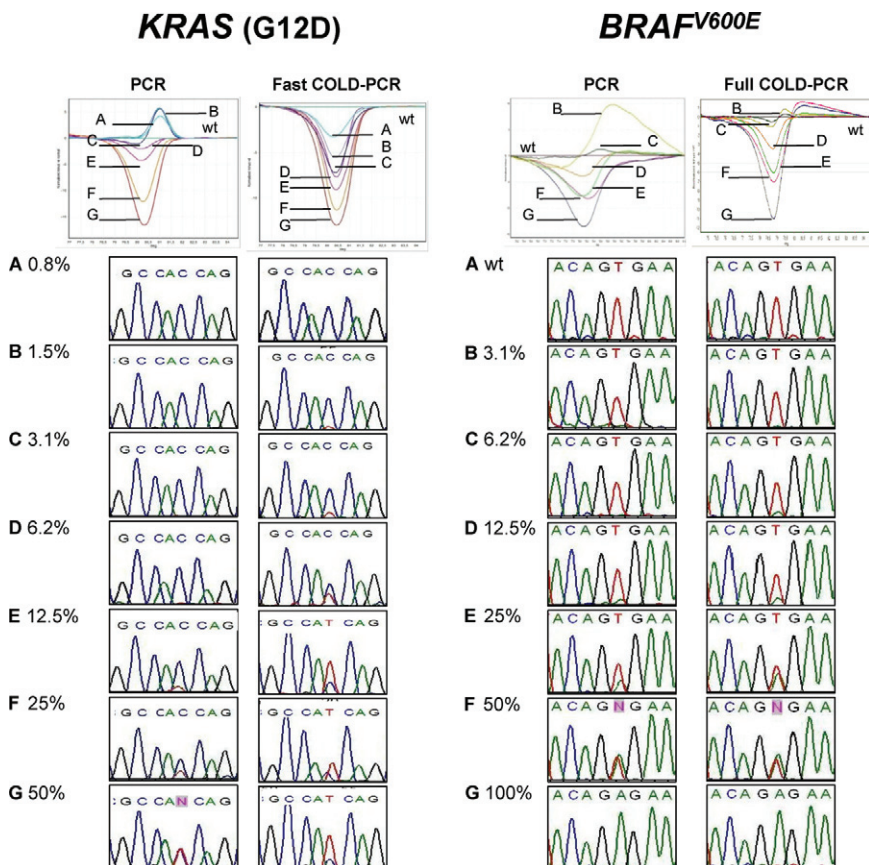
Figure 1. Sensitivity PCR and COLD-PCR for a *KRAS* mutation (G12D) and *BRAF*[V600E]. **Left:** Dilution tests were performed on serially diluted DNA from a *KRAS*-mutated cell line CCRF-CEM and wild-type DNA from MCF-7 cell line, to obtain **A** = 0.8%, **B** = 1.5%, **C** = 3.1%, **D** = 6.2%, **E** = 12.5%, **F** = 25%, **G** = 50%. Dilutions were submitted to conventional PCR (**left**) and fast COLD-PCR (**right**). After HRM, linearity and sensitivity were compared through differential plot analysis. Direct sequencing of corresponding reconstituted samples are also reported (reverse strand sequence). **Right:** Dilution tests were performed on serially diluted DNA from a *BRAF*-mutated cell line SK-Mel-28 and wild-type DNA from MCF-7 cell line. Mutated DNA was diluted with wild-type DNA: **A** = wild-type, **B** = 3.1%, **C** = 6.2%, **D** = 12.5%, **E** = 25%, **F** = 50%, **G** = 100%. Dilutions were submitted to conventional PCR (**left**) and full COLD-PCR (**right**). After HRM, linearity and sensitivity were compared through differential plot analysis. Direct sequencing of corresponding reconstituted samples are also reported.

seconds and 72°C for 1 minute; final extension at 72°C for 15 minutes.

### High-Resolution Melting Analysis and Sequencing

After conventional PCR or COLD-PCR protocols for *KRAS* and *BRAF* genes, all samples were submitted to HRM in a RotorGene 6000 (Corbett Research) with the following denaturation profile: 5 minutes at 95°C, 1 minute at 40°C and a melting profile from 74°C to 85°C using a ramping degree of 0.05. Sequencing analysis was performed in samples after purification with Qiagen PCR Purification Kit. A cycle sequencing reaction was performed with 2 μl of BigDye Terminator Ready Reaction Mix (Applied Biosystems), and the same primers used in PCR but with a concentration of 0.16 μmol/L in a final volume of 10 μl.

After a second purification with a DyeEx 2.0 Spin Kit (Qiagen), samples were then analyzed with the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Results

Sensitivity of HRM COLD-PCR

To evaluate the theoretical sensitivity of our method, serial dilutions of *KRAS*-mutated (CCRF-CEM) and wild-type (MCF-7) DNA were prepared to obtain reconstituted samples containing 50, 25, 12.5, 6.2, 3.1, 1.5, and 0.8% of mutated alleles. These samples were submitted to conventional PCR and fast COLD-PCR protocols, respectively. The sensitivity was then evaluated by HRM analysis and sequencing. After conventional PCR, 6.2% mutated alleles were detectable in HRM differential graphs,
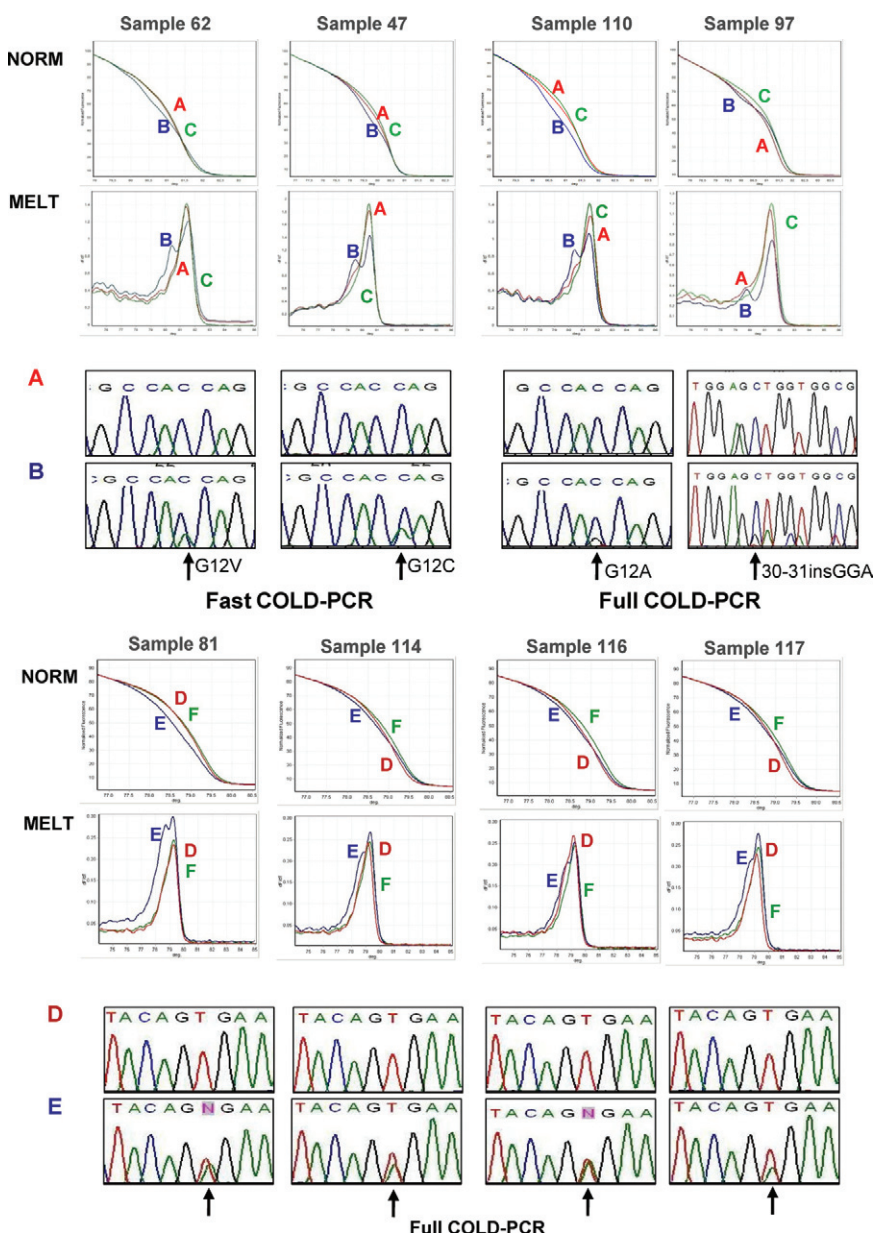
Figure 2. Top: Comparison between conventional PCR and COLD-PCR for *KRAS* mutations in CRC samples. Four examples of CRCs submitted to HRM and sequencing after either a traditional PCR protocol or COLD-PCR. Starting from the upper panels: Norm, HRM profile of a wild-type DNA control (**C**), unknown sample amplified by conventional PCR (**A**), and the same sample amplified by either fast or full COLD-PCR protocols (**B**); Melt, Melt analysis of the same samples of NORM panels. A double peak is evident for all samples submitted to COLD-PCR (**B**) but not for the same samples amplified with conventional PCR (**A**). **A** and **B:** Direct sequencing of the same samples, showing the appearance of *KRAS* mutations (indicated by the **arrows**) in COLD-PCR amplified samples. **Bottom:** Comparison between conventional PCR and COLD-PCR for *BRAF*[N600E] mutation in CRC samples. Four examples of CRCs submitted to HRM and sequencing after either a traditional PCR protocol or COLD-PCR. Starting from the upper panels: Norm, HRM profile of a wild-type DNA control (**F**), unknown sample amplified by conventional PCR (**D**), and the same sample amplified by full COLD-PCR protocol (**E**); Melt, melt analysis of the same samples of NORM panels. A double peak is evident for all samples submitted to COLD-PCR (**E**) but not for the same samples amplified with conventional PCR (**D**). **D** and **E:** Direct sequencing of the same samples, showing the appearance of *BRAF*[V600E] mutations (indicated by the **arrows**) in COLD-PCR amplified samples.

whereas after COLD-PCR 0.8% were easily detectable, indicating an eightfold increase in sensitivity (Figure 1).

Direct sequencing was a less sensitive technique for detecting low abundant variant sequences. The electropherograms of amplification products for *KRAS* obtained with conventional PCR indicated that the minimum detectable percentage was about 12.5%. Conversely, sequence analysis after COLD-PCR allowed the detection of 3.1% mutated alleles, providing a fourfold increase in the detection of this mutant (Figure 1).

The same procedure was adopted to evaluate the theoretical sensitivity of full COLD-PCR in detecting *BRAF*[V600E] in reconstituted samples. HRM was able to detect 12.5% *BRAF*[V600E] after conventional PCR. This percentage was improved by COLD-PCR that allowed to detected until 6.2% of *BRAF*[V600E]. After direct sequencing in PCR products, 25% *BRAF*[V600E] was detectable after conventional PCR and 12.5% after COLD-PCR (Figure 1).

CRC Samples

Initially, screening all of the 117 CRC samples for the research of *KRAS* mutations and *BRAF*[V600E] by HRMA and direct sequencing, we found that 47/117 patients (40.2%) were mutated for *KRAS* and 10/117 (8.5%) for *BRAF*[V600E]. The remaining 70 samples were classified as wild-type.

In a second phase, all samples (mutated and wild-type) were resubmitted to a complete screening using COLD-PCR. Firstly, adopting the fast COLD-PCR protocol to look for *KRAS* variants, we confirmed the presence of genetic variants in the same samples that were classified as positive after PCR and HRM. However, this approach allowed us to detect also eight new *KRAS*-mutated samples. In the 62 samples that were still negative, we used the full COLD-PCR protocol for *KRAS* mutants and were able to enrich and reclassify two CRC samples as mutated. In conclusion, a total of 10/70 (14.3%) new *KRAS*-mutated CRC samples were revealed by COLD-PCR protocols. Examples of conventional and COLD-PCR results are shown in Figure 2 and Supplemental Table 1 (available on *http://jmd. amjpathol.org*).

Similarly, through the full COLD-PCR protocol to look for *BRAF*[V600E], we reconfirmed the presence of 10 mutated samples as already revealed by conventional PCR but identified five new mutated samples initially classified as negative (see examples in Figure 2).

In further five CRC samples (4 for *KRAS* and 1 for *BRAF*), HRM profiles suggested the presence of a possible genetic variant. Unfortunately, we were not able to confirm these findings after direct sequencing (data not shown). This is not surprising taking into account the differences in terms of sensitivity between HRM and sequencing (see previous paragraph). In any case these samples, in the absence of a sequencing confirmation, were classified as not mutated.

Globally, we can affirm that COLD-PCR was able to correctly identify *KRAS* or *BRAF* mutations in 15/70, or

Table 1. List of *KRAS and BRAF* Mutations Detected in Colorectal Cancers Using Conventional and COLD-PCR

Gene	Nucleotide change	Protein mutation	Conventional PCR	COLD-PCR
KRAS	wt		70	60
	c.35G>A	G12D	15	16
	c.35G>T	G12V	9	13
	c.38G>A	G13D	5	7
	c.34G>T	G12C	9	10
	c.35G>C	G12A	3	4
	c.34G>A	G12S	2	2
	c.57G>T	L19F	1	1
	c.37G>A	G13C	1	1
	c.64C>A	Q22K	1	1
	c.30_31insGGA	p.10_11insG	1	2
Total			47	57
BRAF	wt		107	102
	c.1799T>A	V600E	10	15
Total			117	117

21.5% of patients initially classified as negative for the two mutations. See Table 1 for mutations details.

Discussion

Some intrinsic factors can limit the detection of somatic mutations in human cancer biopsies. First, most of the mutations affecting cancer cells are heterozygous. In addition, human solid cancers have a complex cellular architecture in which both normal and pathological cells coexist. Finally, clonal heterogeneity can account for the presence of cellular clones with different mutational status. All these factors can contribute to reducing the percentage of mutated alleles in DNA extracted from tissues, favoring a dilution effect in wild-type DNA. On the other hand, direct sequencing, the reference technique for identification of sequence variants, has a defined detection limit, usually around 20% of mutated alleles. The introduction of pyrosequencing has partially modified this scenario and provides, in the best experimental conditions, as much as 10% sensitivity.[26,32]

To improve identification of low abundant somatic mutations in clinical samples, there are two basic strategies: increasing the sensitivity of the detection systems and enriching the proportion of mutated alleles during PCR amplification.

The first strategy is represented by the introduction of a highly sensitive screening method to identify sequence variants in PCR amplification products, evaluating their temperature-dependent melting profile through HRM. HRM is demonstrated to be a rapid closed-tube post-PCR technique to perform genetic screening in tumor samples. In a recent study[40] we demonstrated the possibility of identifying at least 5% of mutated alleles in a background of wild-type DNA, even if higher sensitivities have been reported by other authors.[22]

The second strategy, indicated as COLD-PCR, enhanced the amplification of low abundant mutated alleles using a specific PCR protocol based on the identification of a critical temperature of DNA denaturation. This enrichment is designed for direct identification of somatic mutations, undetectable after conventional PCR.[31,32] In a

recent study, COLD-PCR was applied to the detection of *KRAS* variants in a heterogeneous group of unselected clinical samples, including 17 paraffin-embedded tissues from primary colorectal cancers and 10 metastases, reporting a mean 1.7-fold increase was obtained.[31] Conversely, COLD-PCR has never been used to enrich *BRAF*[V600E] variants in clinical samples. Very recently the combined use of HRMA and COLD-PCR has been successfully applied to the screening and identification of *TP53* mutations.[33]

In the present study, we focus our interest on detection of *KRAS* or *BRAF* hot spot mutations in CRCs with COLD-PCR and HRM. Our results on reconstituted samples confirm that COLD-PCR is a technique able to enhance sensitivity of screening of mutations of both genes. The increase was particularly evident after HRM analysis, with eightfold higher sensitivity for *KRAS* and fourfold for *BRAF*. The difference in the increment between the two genes is probably due to the different protocol used to test sensitivity: fast-COLD for *KRAS* and full for *BRAF*. When the results of COLD-PCR were confirmed with sequencing, the increased sensitivity was reduced by the innate limitation of this technique.

The increased sensitivity of COLD-PCR for *KRAS* and *BRAF* mutations was also tested in a group of 117 CRCs and compared with results obtained with a standard PCR-HRM protocol.[39] By COLD-PCR we confirmed the presence of mutations in all samples initially classified as mutated using conventional PCR-HRM, and we also identified a significant subset (five *BRAF* and 10 *KRAS* of 117) of CRCs initially classified as negative for both genes. This means that COLD-PCR is able to improve the sensitivity of HRM-PCR, a technique that was already considered particularly sensitive in mutation screening, as proved by the relatively high percentage of mutated samples we initially found (40.2% for *KRAS* and 8.5% for *BRAF*).

It is also important to remark that in a small subset of 5 CRC samples, HRM evidenced an abnormal profile compatible with the presence of a mutant in the sequence not confirmed by direct sequence analysis. Due to cited difference of sensitivity of the two techniques (from 5% of HRM to the 20% of sequencing), we can postulate that few samples with a very low-level mutation can fall, after COLD-PCR, in that window detectable with HRM but not with sequencing. Further research in this matter is in progress in our laboratory.

Taking our population study as a model, we can conclude that by combining COLD-PCR and HRM, we were able to detect *KRAS* and *BRAF* mutations in about 25% of patients initially classified as wild-type CRCs and, according to our strategy, the percentage of patients with either *KRAS* or *BRAF* mutations passes from 48.7% to 61.5%. To understand the real value of this 12.8% increase of samples correctly classified for *KRAS* and *BRAF* mutations we must consider that there were 150,000 newly diagnosed colorectal cancer patients in the United States in 2009 (*http://seer.cancer.gov/csr/1975_2006/*). This means that, in theory, about 19,000 of these patients could be correctly classified for *KRAS* or *BRAF* mutation, using HRM COLD-PCR.

In conclusion, the correct identification of less-represented mutations in CRC can be significantly improved with COLD-PCR combined with HRM, without requiring expensive and time-consuming procedures and while maintaining a closed-tube approach. HRM COLD-PCR has the potential to improve the routine search for mutations in cancer tissues. It is important to remark that today the real significance of low-level KRAS and BRAF mutations remains to be clinically proven, mainly for their predictive role in response to targeted therapy.

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