High-resolution melting for detecting *KRAS* mutations in colorectal cancer

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Abstract. Colorectal cancer is a leading cause of cancer that may metastasize. *KRAS* gene sequence of exon 2 should be examined for identification of patients that can be treated with anti-EGFR. The aim of the present study was to evaluate the efficacy of high-resolution melting (HRM) to detect *KRAS* mutations in colorectal cancer (CRC) tumors. The exon 2 of KRAS was amplified from 47 adenocarcinoma CRC tissues. The tumors were subjected to high-resolution melt using quantitative PCR to identify wild-type and mutant subgroups. The results were compared to the mutations detected by next-generation sequences (NGS). The study included 47 patients, with a mean age of 62 years, of whom 24 patients were male. Most of the patients had stage II or stage III tumors. The mean melting temperatures for the wild-type and mutated group at exon 2 were 78.13˚C and 77.87˚C, respectively (P<0.001, 95% CI = 0.11-0.4). The sensitivity and specificity of high-resolution melting were 83.3 and 96.6%, respectively, with a high concordance between the NGS and HRM methods for detecting KRAS mutation in exon 2 (κ = 0.816; P=0.625). Thus, HRM could be used as an alternative method for detecting KRAS mutations in colorectal cancer tissue.

Introduction

Colorectal cancer (CRC) is the third most common cancer in Brazil and globally (1,2). The most prevalent molecular pathways for colorectal cancer development are *APC*, *TP53*, and *KRAS* mutations. The *KRAS* gene is 47,305 bp long and contains 6 exons. It plays the role of a GTPase in the transduction of signals (3). The activation of *KRAS* forms a GTP complex, which can then be inactivated by hydrolysis to GDP. The mutated form of KRAS renders the complex less susceptible to hydrolysis, remaining in an activated form which induces the cell to proliferate via several signal pathways, including MAPK (3,4).

The RAS family includes three subunits: Kirsten-RAS (KRAS), Neuroblastome-RAS (NRAS), and Harvey-RAS (HRAS). Mutations of KRAS are found in 34.7%, of NRAS in 7%, and of HRAS in 0.5% of CRC. Mutations in *KRAS* and *NRAS* confer a poor prognosis, even at the metastatic stage or early stage colorectal cancers. Additionally, they can lead to the development of resistance against anti-EGFR molecules (5-9). By contrast, EGFR inhibitors confer a positive predictive value response and increased overall survival in KRAS and NRAS wild-type tumors (10). Venook *et al* suggested that sidedness of the primary tumor greatly affects the clinical outcomes in an advanced or metastatic setting (11). The median survival for primary tumors located on the left side was significantly longer than that for tumors of the right side (overall survival: 33.3 vs. 19.4 months; P<0.0001). Patients treated with cetuximab with wild-type KRAS and left-sided primary tumors had an OS of 37.5 months versus those with right-sided primary tumors who had an OS of 16.4 months (HR = 1.97; 95% CI = 1.56-2.48). This suggests that patients with primary tumors on the right side of the colon should not be treated with anti-EGFRs (11).

DNA sequencing is the gold standard for detecting mutations. Originally, Sanger sequencing constituted standard usage; however, next-generation sequencing (NGS) has allowed for faster and high-throughput screening for mutations in several types of cancer (12). High-resolution melting (HRM) has recently been used as an alternative strategy to DNA sequencing (13). Through differences in DNA melting temperatures and curve profiles, it is possible to distinguish mutant samples from controls. HRM analyzers allow for a more accurate detection of differences in melting temperatures between two samples (13). In the present study, it was hypothesized that HRM could be used as an effective alternative to next-generation sequencing for the detection of KRAS mutations in colorectal cancer.

Materials and methods

General. The present study was performed in accordance with the Declaration of Helsinki and approved by the Universidade Federal de Sao Paulo Ethics Committee Plataforma Brasil CAAE: 55446116000005505, Biobank BR080. All the patients

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signed an informed consent allowing tumor samples to be used in our study. The study included 47 patients, with a mean age of 62 years. Of the 47 patients, 24 were male.

A 25 mg sample from each confirmed colorectal adenocarcinoma was collected for DNA sequencing using the Illumina HiSeq 2500 Platform and for DNA analyses for HRM via the StepOne Plus® Real-Time PCR Systems.

In addition, using a second-generation Illumina DNA sequencing platform as a reference, we compared the HRM capacity to identify mutations in exons 2, 3, and 4 of KRAS. A total of 47 fresh-frozen tissue samples were obtained.

DNA extraction and quantification. DNA was extracted from 25 mg of fresh-frozen colorectal adenocarcinoma tissue using the QIAmp® DNA Mini Kit (Qiagen®) according to the manufacturer's instructions. Samples were cut into small fragments, incubated at 56˚C for 6 h, and vortexed every 30 min. DNA was eluted in nuclease-free water and quantified by spectrophotometry (NanoDrop®; Thermo Fisher Scientific) and fluorimetry (Qubit®; Thermo Fisher Scientific) and stored in cryotubes at -20˚C. Every 25 mg of the tumor sample yielded approximately 30 μg of DNA.

Quantitative PCR and analysis of DNA melting temperatures. Quantitative PCR with the StepOne Plus® Real-Time PCR Systems (Applied Biosystems), using Meltdoctor™ HRM Master Mix (Applied Biosystems) and forward (f) and reverse (r) primers of exon 2 of KRAS, was carried for DNA amplifications and post-PCR melt analyses. Exon 2f: TTA TAA GGC CTG CTG AAA ATG ACT GAA; exon 2r: TGA ATT AGC TGT ATC GTC AAG GCA CT. The PCR assays were carried out in a final reaction volume of 25 μl containing 12.5 μl of Meltdoctor™, 1 μl each of forward and reverse primers at 10 pmol/ml, respectively, and 1 μl of DNA with a concentration range of 20-100 ng. The PCR conditions were: denaturation at 95˚C for 10 min, followed by 50 cycles at 95˚C for 15 sec, annealing at 62˚C for 10 sec, and an extension at 72˚C for 10 sec. For the HRM assays, we performed denaturation at 95˚C for 15 sec, and obtained high-resolution melting profiles from 60 to 95˚C at intervals of 0.5˚C. All the HRM reactions were performed in triplicate. After the PCR runs, the melting curve profiles were analyzed by HRM Software v3.01 (Thermo Fisher Scientific).

DNA next-generation sequencing. NGS for exon 2 was conducted using the TruSight™ Tumor Sample Preparation Kit (Illumina®; San Diego) and the TruSight™ Tumor 26 (Illumina®). The steps were performed according to the manufacturer's recommendations. These included: hybridization of the oligo pool for 15 min and incubation for 10 h, removal of unbound oligos for 20 min, extension-ligation of bound oligos for 5 min and incubation for 45 min, PCR amplification, PCR cleanup, library quantification and normalization, and library denaturing and pooling. To identify the somatic mutations, we used the Catalogue of Somatic Mutations in Cancer (COSMIC, www.cancer.sanger.ac.uk). Patients were divided into wild-type and mutant groups as per the results of NGS.

Statistical analysis. Statistical analyses were performed using SPSS software v.21 (SPSS, Inc.; Chicago, IL, USA). The κ coefficient with a 95% CI was used to assess the agreement between the two methods. A t-test for independent samples was carried out to compare the mean melting temperatures between wild-type and mutant groups, and the McNemar test was used to describe the sensitivity and specificity of HRM at exon 2 for the *KRAS* mutations.

Table I. Patient characteristics.

Characteristic	N / mean (range)	(%)
Sex		
Female	23	48.9
Male	24	51.1
Age	62.39 (35-86)	-
Pathological features		
Mucinous	9	19.1
Well-differentiated	12	25.5
Poorly differentiated	3	6.3
Moderately differentiated	18	38.3
Perineural invasion	15	31.9
Vascular invasion	18	38.3
Inflammatory process	10	21.2
Staging		
I	1	2
II	24	50
III	14	29.2
IV	5	10.4

Results

Patient characteristics. Forty-seven patients were included, the mean age was 62 years and 24 were males. The pathological features included mucinous characteristics (19%); well-differentiated (25.5%), and poorly differentiated (6.3%). Most of the patients had stage II or III tumors. Table I summarizes the baseline characteristics of the patients.

The tumors were sequenced on the HiSeq Sequencing Platform and then subjected to PCR amplification and DNA melting.

Next-generation sequencing. In total, 16 tumors (34%) had mutations in *KRAS*; 78% of these mutations were in exon 2, 11% in exon 3 and 11% in exon 4. The mutation in exon 2 of *KRAS* included c.35C > T (G12D); c.34C > A(G12C), and c.38C > T(G13D) (Fig. 1).

High-resolution melting. The mean melting temperatures for the wild-type and mutated group at exon 2 were 78.13˚C and 77.87˚C, respectively (P=0.001, Table II). The melting curve profiles are shown in Figs. 1 and 2.

Sensibility and specificity for HRM. The sensitivity and specificity of high-resolution melting were 83.3 and 96.6%, respectively, with a high concordance between the methods (κ =0.816; P=0.625) (Table III). Of the 16 samples diagnosed

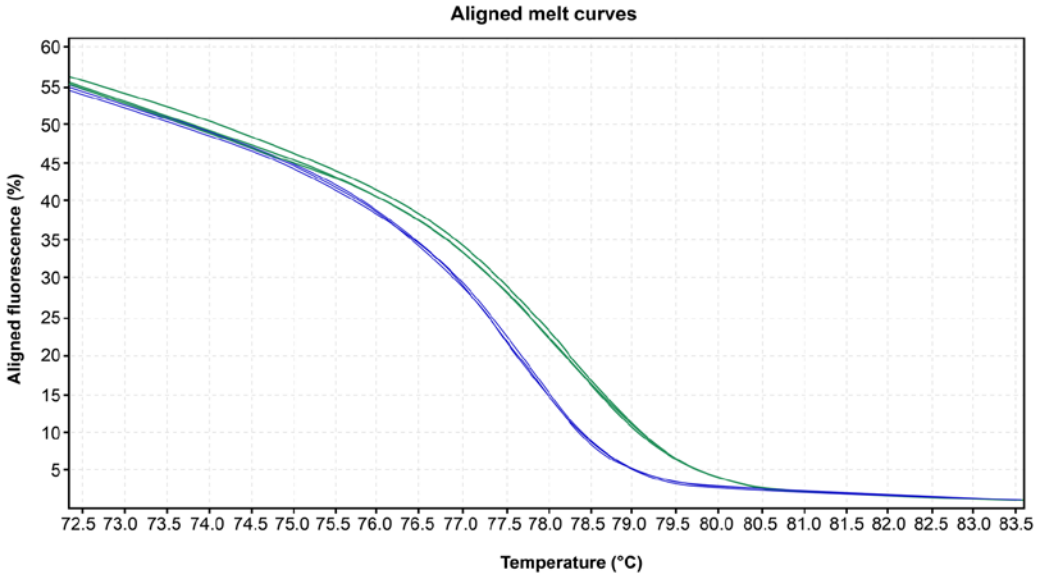

Figure 1. Aligned melt curves for exon 2 of KRAS. Green, wild-type group; Blue, mutant group.

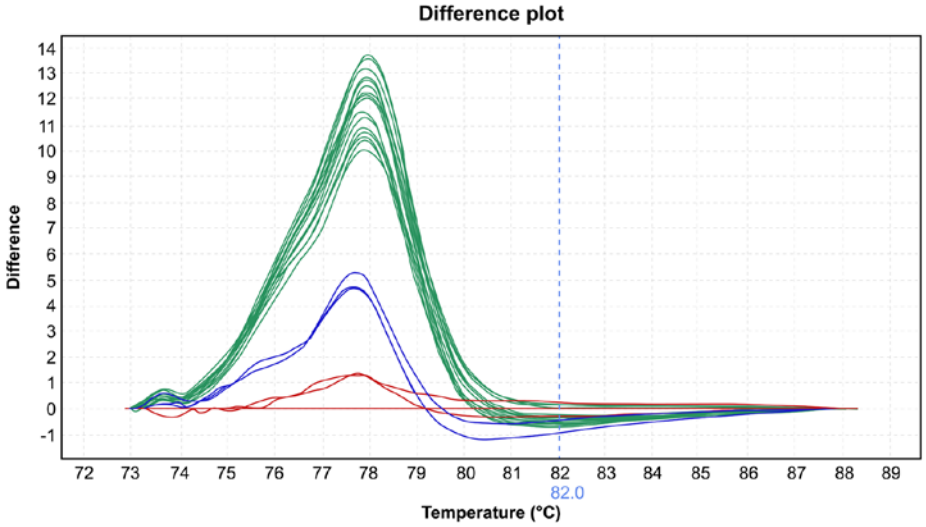

Figure 2. Difference plot for exon 2 of KRAS showing three different patterns of melting curve. Green, wild-type; Blue, c.35 C>T(G12D); Red, c.43 C>A(G15C).

Table II. Mean temperature (˚C) of melting for the wild-type and mutated groups according to next-generation sequencing.

Item	Wild-type	Mutated	95% CI	P-value
Mean ± SD	78.1352±15	77.87±0.28	0.11-0.41	0.001

Table III. Mutation on KRAS exon 2 by HRM and NGS.

	HRM		
NGS	Wild-type	Mutated	P-value[a]
Wild-type [N (%)]	26 (96.6)	1 (3.4)	0.625
Mutated [N (%)]	3 (16.7)	15 (83.3)	

[a]κ coefficient of concordance = 0.816. HRM, high resolution melting; NGS, next-generation sequence; WT, wild-type.

as mutant by HRM, one was not considered mutated by NGS. Furthermore, we identified 29 samples as wild-type via HRM and one of those samples was considered mutated via sequencing.

Discussion

Personalized oncology claims that biomarkers predict a likely response to chemotherapy. In CRCs, somatic mutations in exon 2 of *KRAS* are predictive for resistance to anti-EGFR antibodies. Several studies have reported mutations in exon 2, ranging from 36 to 45% (14). Next-generation sequencing, which

demonstrates a high concordance with Sanger sequencing methods, has been used for faster detection of mutations in different genes within larger DNA sequences (15).

Ishige *et al* reported that 25% of the mutations in exon 2 were in codon 12 and 4% in codon 13 (6). Neumann *et al* (16) also reported that 80% of the mutations in exon 2 were in codon 12. In the present study, the prevalence of *KRAS* mutations was 34%, mainly in exon 2. The mutations on exon 2 were 12% in codon 13, and 54% in codon 12. Although findings of those studies lacked concordance, they did agree that codon 12 at exon 2 is the most common site of mutation in *KRAS* for colorectal cancer.

High-resolution melting has been used as an alternative strategy for sensitive detection of DNA sequence variations in different genes (17). This method is based on the different DNA melting temperatures for specific targets. This method cannot, however, be used to identify changes in the DNA sequence, although wild-type sequences and mutant sequences correlate with specific, and different, melting curves. In CRC, HRM has been evaluated to validate its routine use compared with both direct sequencing and NGS in various studies (18-23).

The HRM assay with DNA concentrations ranging from 20 to 100 ng, were able to evaluate the exon 2 of *KRAS* with a sensitivity and specificity of 83.3 and 96.6%, respectively, compared to NGS. Demonstrating a high concordance between the two methods used ($\kappa = 0.816$). Negru *et al* reported concordance of approximately 99% between HRM and DNA sequencing for exon 2 (20). The large capacity for detecting mutations in exon 2 of *KRAS* in our study was similar to that in other studies with different populations (17,24-26).

The short number of samples is a limitation of this study. However, this study proved that this technique had a high sensitivity and specificity that may be validated in a high number of tumors. This assay, different from the others that was carried out in formalin-fixed paraffin-embedded (FFPE) tissue, was elaborate in fresh tumor samples that can increase the sensitivity of detection of mutations. A possible explanation is a possible DNA degeneration during the preparation and maintenance of the archived samples in FFPE. Another possible advantage when compared to other published articles is that the sequencing of *KRAS* mutation was by NGS and not by Sanger or pyrosequencing, which both have a low sensitivity.

Two advantages had to be considered in the diagnosis of *KRAS* mutations in CRC by HRM. This method is less expensive than NGS and may be an alternative method to diagnose *KRAS* mutation This is particularly important in view of the growing need to identify mutant profiles for different target genes in many types of cancer. Another advantage of HRM is the time spent for performance of the test, i.e., approximately 3.5 h compared to NGS, which requires some days.

In conclusion, in our cohort, HRM had a high index of comparison with NGS, which was the gold standard for detecting DNA mutations of *KRAS* in colorectal cancer.

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Availability of data and materials

The datasets generated and analyzed in the present study are included in this published article.

Authors' contributions

RRM contributed to performance of the experiments of HRM, statistical analysis and writing the article. TDS contributed to acquisition of tumor samples, extraction of DNA and DNA sequencing. NMF contributed to the concept, design and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Resolution 466 of December 08, 2012, of the National Health Council of Brazil. Ethical approval for this study was obtained from the Ethics and Clinical Research Committee Coordenadoria de Ensino e Pesquisa do Hospital São Paulo-HU/UNIFESP. CAAE: 55446116000005505. Patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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