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Evaluation of High-Resolution Melting Analysis as a Diagnostic Tool to Detect the *BRAF* V600E Mutation in Colorectal Tumors

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BRAF* V600E is the predominantly occurring mutation of the cytoplasmic kinase *BRAF*, and, in colorectal cancer, its determination provides a diagnostic exclusion criterion for hereditary nonpolyposis colorectal cancer. The aim of our study was to develop a sensitive *BRAF* V600E high resolution melting (HRM) assay. We first established and optimized the *BRAF* HRM assay using a cell line dilution model, enabling us to detect 1% mutant DNA in a background of wild-type DNA. In a comparison, DNA sequencing and real-time allele-specific PCR in the cell line dilution model HRM assay proved to be more sensitive than DNA sequencing and denaturing high performance liquid chromatography, retaining the same sensitivity as real-time allele-specific PCR. In a learning set of 13 patients with known *BRAF* V600 status, the mutation was detected with high concordance by all four methods. Finally, we validated the HRM assay on 60 formalin-fixed, paraffin-embedded colorectal cancer samples. Although all mutated samples were correctly identified by HRM, the detection limit of the HRM assay decreased when using low-quality DNA derived from formalin-fixed, paraffin-embedded samples. In conclusion, HRM analysis is a powerful diagnostic tool for detection of *BRAF* V600E mutation with a high sensitivity and high-throughput capability. Despite the expected decrease in sensitivity, HRM can reliably be applied in archival formalin-fixed, paraffin-embedded samples tissues.** ***(J Mol Diagn 2009, 11:140–147; DOI: 10.2353/jmoldx.2009.080100)

The cytoplasmic localized serine/threonine kinase *BRAF* is a part of the RAS/RAF/MAPK signal transduction pathway, which acts as a major mediator for cell growth in different human cells. An oncogenic hotspot mutation in the *BRAF* gene, the *BRAF* V600E mutation in exon 15, has been described in a variety of human cancers, especially melanoma, thyroid cancer, and colorectal cancer.[1,2] In colorectal cancer, mutated *BRAF* kinase contributes to carcinogenesis by increasing resistance to apoptotic stimuli and promoting development of invasive phenotype.[3] *BRAF* mutations have been associated with a poor survival in colon cancer.[4,5] Given that the *BRAF* V600E mutation is exclusively found in malignant cells, this alteration provides a novel target for anticancer therapeutics, and *BRAF* inhibitors are currently under clinical investigation.[6,7] In addition to the use of *BRAF* V600E as a potential predictive factor, detection of *BRAF* V600E mutation has been proposed as a diagnostic marker to distinguish sporadic microsatellite instable colon cancer from hereditary non-polyposis colorectal cancer (Lynch syndrome). The presence of *BRAF* V600E suggests a sporadic origin of microsatellite instable colorectal cancer and detection of this mutation, therefore has a potential to be used before time-consuming and expensive hereditary non-polyposis colorectal cancer testing.[8,9] Until now, a variety of methods for detection of this mutation in colorectal cancer has been described, including single-strand conformation analysis, DNA sequencing, TaqMan-based real-time PCR, real-time allele-specific PCR, pyrosequencing, and oligonucleotide microarray.[9–15] DNA sequencing, currently the gold standard for mutational analysis, is limited by high cost and low sensitivity. Furthermore, most of the mentioned assays are time-consuming and require manipulation of amplified PCR products, which is a common source of sample contamination. Pyrosequencing offers a sensitive alternative method, but the equipment is expensive and may not be economical for low-throughput laboratories. For Taqman-

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based real-time PCR analysis the major disadvantage is the need for expensive fluorescence-labeled probes.

High resolution melting (HRM) analysis is a recently developed molecular technique proved to be applicable for detection of various clinically relevant human mutations.[16–21] It is a cost-efficient, closed-tube system that allows high-throughput analysis without any post-PCR processing, features that are desired characteristics for research and clinical application. The aim of our study was to develop a clinically useful *BRAF* V600E HRM assay and to critically compare the HRM-based assay with three commonly used mutation detection techniques. We used a cell line-based dilution model to establish and optimize a *BRAF* V600E HRM assay and, in a second step, compared the sensitivity of HRM with denaturing high performance liquid chromatography (DHPLC), real-time allele-specific PCR, and DNA sequencing. Finally, we validated the HRM assay using a collection of archival formalin-fixed paraffin-embedded (FFPE) colorectal cancer samples.

Materials and Methods

Cell Lines and Patient Samples

For optimization of the four different mutation detection methods, we used a cell line model system consisting of two human colon cancer cell lines (HT29 and SW480). The HT29 cell line harbors the heterozygous *BRAF* V600E mutation, and SW480 is homozygous for the wild-type allele. Cell lines were grown under standard conditions and genomic DNA was extracted using the Blood and Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To optimize the sensitivity of the *BRAF* HRM assay, we used DNA extracted from HT29 mixed with DNA obtained from SW480 at the following dilutions: 100%, 20%, 10%, 5%, 1%, and 0% of tumor cell line DNA known to harbor *BRAF* mutation. For comparison of the analytical sensitivity of all four methods, the same dilutions were used. Results obtained by each method were assessed independently by three different investigators in a blinded fashion.

After completing the cell line experiments, we validated the *BRAF* HRM assay for its ability to detect the *BRAF* V600E mutation on routinely used FFPE blocks. Ethical approval for this research was obtained from the local ethical committee. DNA analysis was performed in two independent cohorts. The learning set consisted of 13 FFPE colon cancer tissues showing negative immunohistochemical staining for hMLH1 where 11 tissues were known to harbor *BRAF* V600E mutation. The training set involved 60 randomly selected colorectal carcinoma patients, ages 41 to 87 years (median 67), with histologically confirmed colorectal cancer. In the training set staging had been performed according to the International Union Against Cancer guidelines. There were 17 stage I, 11 stage II, 20 stage III, and 12 stage IV colorectal carcinomas. In all of the cases tumor areas were marked on the H&E-stained slides and corresponding unstained slides were microdissected so that samples contained at least 50% tumor cells. DNA was isolated from scraped material as described by Wu et al.[22] DNA quantity was assessed spectrophotometrically and quality of genomic DNA was confirmed by agarose gel electrophoresis.

Where corresponding fresh frozen samples were available, we additionally analyzed DNA from cryopreserved tissue. DNA was extracted according to the QIAamp DNA micro kit protocol (Qiagen) and isolated DNA was quantified spectrophotometrically and stored at −20°C.

In an additional step, we evaluated the sensitivity of the HRM in FFPE samples. After the analysis of the training set was completed, we selected one sample where the *BRAF* V600E mutation was detected, and two independent wild-type samples. We prepared the same dilutions as in the cell lines. The *BRAF* V600E positive sample was mixed with the wild-type DNA at 100%, 20%, 10%, 5%, 1%, and 0%, respectively.

High Resolution Melting Analysis

PCR and HRM were consecutively done on a LightCycler 480 (Roche Diagnostics, Vienna, Austria) in one single run, and all reactions were performed in triplicate. Primers were selected to flank the *BRAF* V600E mutation. We evaluated two different amplicons for the ability to detect *BRAF* mutation. Primers for the 238 bp amplicon were Fw 5′-CATAATGCTTGCTCTGATAGGAAA-3′ and Rv 5′-TCAGCACATCTCAGGGCCAAA-3′ (primer sequences were obtained at May 2, 2008 from *http://www.mutationdiscovery.com/md/MD.com/home_page.jsp*) and for the shorter 147 bp amplicon were Fw 5′-GGTGATTTTGGTCTAGCTACAG-3′ and Rv 5′-AGTAACTCAGCAGCATCTCAGG -3′ (primers were designed with Primer3 software[23]). The melting behavior of the smaller 147-bp amplicon was designed to contain only a single melting domain by using Poland software.[24] Each reaction mixture contained 1 μl of DNA solution (50 ng), 200 nmol/L of each primer, 10 μl of LightCycler LC480 High Resolution Melting Master (Roche), 3 mmol/L $MgCl_2$, and water to a final volume of 20 μl. PCR conditions were: 95°C for 10 minutes, followed by 45 cycles of 10 seconds at 95°C, a touchdown of 64°C to 54°C for 10 seconds (1°C/cycle), and 20 seconds at 72°C. After amplification, the PCR product was denatured at 95°C for 1 minute and cooled down to 40°C to allow heteroduplex formation. The final HRM step was performed from 75°C to 95°C with an increase of 1°C per second with 25 acquisitions per degree. The HRM curve analysis was performed using the Gene Scanning Software (Roche). For sample analysis, melting curves were normalized, temperature-adjusted and, finally, a difference plot was generated. A wild-type control (ie, DNA from the wild-type cell line SW480) was used to normalize melting profiles of the other samples against this predefined horizontal baseline. Samples were considered mutated when significant difference of fluorescence level for all triplicates fell outside of the range of variation detected for the wild-type control.

To evaluate the influence of poor quality DNA from FFPE samples on the melting behavior of the amplicon,