Disease Markers 34 (2013) 171–177
DOI 10.3233/DMA-120960
IOS Press

Duplex reverse-hybridization assay for the simultaneous detection of *KRAS/BRAF* mutations in FFPE-extracted genomic DNA from colorectal cancer specimens

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Abstract. We report the performance evaluation of a non-quantitative reverse-hybridization assay (*KRAS-BRAF* StripAssay) designed for the simultaneous detection of 10 mutations in codons 12 and 13 of the *KRAS* gene and *BRAF* mutation V600E. Dilution experiments using DNA from tumor cell lines or from formalin-fixed paraffin-embedded (FFPE) colorectal cancer (CRC) tissue were performed to assess assay sensitivity. Using 50 ng of total DNA (mutant and wild-type), the *KRAS-BRAF* StripAssay demonstrated a detection limit of 1% mutant sequence in a background of wild-type DNA. With respect to *BRAF* V600E, the *KRAS-BRAF* StripAssay was evaluated using 60 FFPE CRC samples previously analyzed by high resolution melting (HRM). Test strip hybridization identified 2/60 (3%) samples to carry the *BRAF* V600E mutation, and results were in agreement with those obtained by HRM analysis. This work demonstrates the *KRAS-BRAF* StripAssay to be a robust and sensitive method for the detection of common *KRAS*/*BRAF* mutations in genomic DNA isolated from FFPE tissue samples.

Keywords: *KRAS*, *BRAF*, mutation detection, reverse-hybridization, high-resolution melting

1. Introduction

Testing for *KRAS* mutation is an accepted prerequisite to select patients with colorectal cancer (CRC) for epidermal growth factor receptor (EGFR)-targeted therapies; typically, only individuals with wild-type *KRAS* respond to anti-EGFR therapy [1]. However, *KRAS* mutations account for approximately 35–45% of non-responders, only [2]. Relatively recently additional activating mutations in EGFR downstream effectors, such as the BRAF kinase, have been identified [3]. *BRAF* V600E (a thymidine to adenine transversion at nucleoside 1799 in exon 15) is the most prominent among more than 40 known *BRAF* mutations leading to a valine to glutamate transversion at residue 600 and constitutive kinase activity [3]. Overall, *BRAF* belongs to the commonest oncogenes mutated in 7–9% of all solid malignant tumors [4], and is well known to be present already in benign lesions, such as ser-

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ISSN 0278-0240/13/$27.50

rated colorectal adenoma, benign nevi, and indolent micro-papillary thyroid cancers (PTCs) [4–6]. Thus *BRAF* mutation might be a founder event in carcinogenesis, but by itself is not sufficient to induce cancer, as only mutations secondary to *BRAF* can accelerate the growth to clinically evident disease [4,7]. *BRAF* mutation occurs in 10–18% of CRC overall, but is more frequently encountered in microsatellite instable carcinomas (MSI) with preferentially MLH1 promotor hypermethylation and CpG island methylation phenotype (CIMP) [8,9]. Patients with *BRAF* V600E colorectal tumors show impaired responsiveness to the anti-EGFR therapeutic antibodies cetuximab and panitumumab [10]. Because *KRAS* and *BRAF* mutations are mutually exclusive in CRC [11], *BRAF* V600E testing may further empower the selection of patients eligible for anti-EGFR antibody treatment [2]. In conjunction with the very recently described *PI3KCA* activating mutations and the inactivation of PTEN phosphatase (a PI3K inhibitor), Bardelli et al. established the term "quadruple negative" tumors as having the highest probability of response to anti-EGFR therapies [2].

Melanoma is the malignancy with the highest *BRAF* mutation frequency ranging from 40–70%; V600E accounts for 95% of all *BRAF* mutations, thereby playing an important role in tumor progression in cooperation with PI3K-induced signaling [12].

In PTC mutant *BRAF* is found in 29–87% of all cases. As PTC possesses a very favourable overall prognosis, the value of *BRAF* as a molecular prognostic marker is still under discussion [4].

We have recently evaluated a non-quantitative reverse-hybridization assay (*KRAS* StripAssay) for the simultaneous detection of 10 frequent mutations in codons 12 and 13 of the *KRAS* gene [13]. The *KRAS* StripAssay has an analytical sensitivity of 1% and has been shown to be fully compatible with *KRAS* mutation analysis in genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue [13–15].

Here we present an extended version of the *KRAS* StripAssay that has been modified to contain also the *BRAF* V600E mutation (*KRAS-BRAF* StripAssay). We evaluated the novel *KRAS-BRAF* StripAssay by comparing it with a high resolution melting (HRM) protocol designed for the sensitive detection of *BRAF* V600E (*BRAF* HRM assay). Recent work used the *BRAF* HRM assay to screen for mutation V600E in 60 FFPE tissue samples obtained from CRC patients [16]. The same samples were then analyzed with the *KRAS-BRAF* StripAssay, and results were compared with respect to sensitivity, specificity and utility.

2. Patients and methods

2.1. Patient samples

FFPE-extracted DNA samples were available from 60 randomly selected patients aged 41 to 87 years (median 67) with histologically confirmed CRC. Staging was performed according to the International Union Against Cancer (UICC) guidelines including 17 stage I, 11 stage II, 20 stage III, and 12 stage IV tumors. An appropriate paraffin block containing tumor tissue was selected for analysis after reviewing the hematoxilin-eosin (HE) stained slides. An area of tumor on the HE stained slide was marked and microdissected on a corresponding unstained slide so that samples contained at least 50% tumor cells. All DNA samples had previously been screened for *BRAF* V600E mutation status by HRM and sequence analysis [16]. Patients gave their written informed consent and the study was approved by the local ethical committee.

2.2. Cell lines and DNA dilutions

Real-time PCR was used to quantitate DNA from tumor cell lines as well as patient DNA samples as described previously [13]. Genomic DNA isolated from tumor cell lines SW480 (*KRAS* Val12), LS174T (*KRAS* Asp12), A549 (*KRAS* Ser12), SW1116 (*KRAS* Ala12), MIA Paca2 (*KRAS* Cys12), H157 (*KRAS* Arg12), H1355 (*KRAS* Cys13), DLD1 (*KRAS* Asp13), HT29 (*BRAF* V600E) and Colo320 (wild-type) served as control templates. In the absence of suitable cell line DNA, controls for *KRAS* mutations Ile12 and Leu12 were generated by site-directed mutagenesis [17]. To monitor assay sensitivity and specificity, PCR was performed on 0.5 ng mutant genomic DNA mixed with 50 ng wild-type DNA and on 50 ng wild-type DNA alone. To evaluate assay sensitivity in FFPE-extracted DNA, clinical samples carrying the *BRAF* V600E mutation were mixed with wild-type DNA at 50%, 10%, 1%, and 0%.

2.3. KRAS-BRAF StripAssay

The *KRAS-BRAF* StripAssay (ViennaLab Diagnostics, Vienna, Austria) was performed as described earlier [13] except for the fact, that the amplification mix contained *BRAF*-specific primers 5'-GGTGATTTTGGTCTAGCTACAG-3'(forward) and 5'-AGTAACTCAGCAGCATCTCAGG-3'(reverse).
Briefly, PCR was done in a 25 μL reaction containing

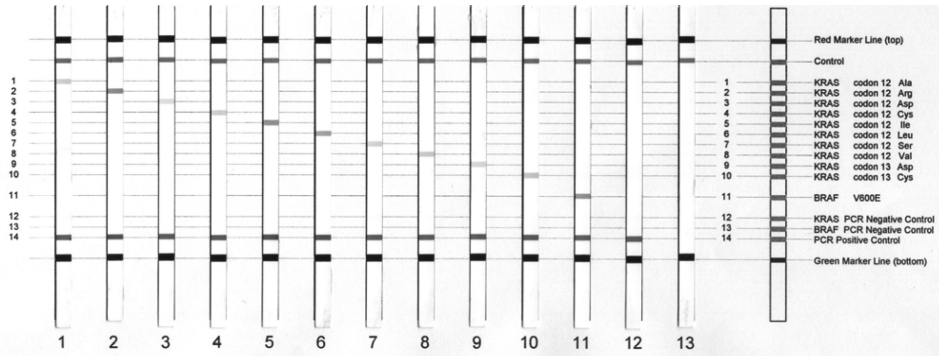

Fig. 1. *KRAS-BRAF* StripAssay analysis of tumor cell lines and mutant DNA plasmids generated by site-directed mutagenesis. Test strips obtained after reverse-hybridization and enzymatic color development are shown. The StripAssay was performed on 0.5 ng mutant genomic DNA mixed with 50 ng wild-type DNA (strips 1–11), and 50 ng wild-type DNA alone (strip 12). PCR non-template control (strip 13).

15 μL amplification mix, 5 μL of a dilution containing Taq DNA polymerase (1U), and 5 μL DNA template (up to 50 ng genomic DNA). The amplification was performed on a PE 9700 thermocycler (Applied Biosystems, Foster City, CA) starting with an initial denaturation step at 94°C for 15 min, then running 35 cycles of 94°C for 1 min, 70°C for 50 sec, 56°C for 50 sec, 60°C for 50 sec, and a final extension at 60°C for 7 min. Biotinylated amplification products were then hybridized to test strips strictly controlling temperature (45 $\pm$ 0.5°C), and bound sequences were visualized using a streptavidin-alkaline phosphatase conjungate and colour substrates. Reverse-hybridization was carried out manually using a shaking waterbath (GFL, Burgwedel, Germany) set to 45°C, and results were interpreted using the enclosed Collector sheet. The operator was blinded to the HRM results.

2.4. Direct PCR sequencing

DNA sequencing was performed as described previously [13]. Briefly, *KRAS*-positive PCR products were column purified using the GenElute PCR CleanUp Kit (Sigma-Aldrich, St. Louis, MO), and sequence analysis was performed on a ABI 310 automatic sequencer (Applied Biosystems) according to the manufacturer's instructions (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) using the *KRAS* sense primer.

3. Results

3.1. Analytical sensitivity

Recently, the *KRAS* StripAssay has been successfully applied to *KRAS* mutation analysis in FFPE-extracted DNA [13–15]. The *KRAS-BRAF* StripAssay under investigation here has been modified to contain 10 mutations in codons 12 and 13 of the *KRAS* gene and *BRAF* mutation V600E. It also combines mutant-enriched PCR based on peptide nucleic acid (PNA) clamping and reverse-hybridization of biotinylated PCR products to nitrocellulose test strips containing a parallel array of oligonucleotide probes. The StripAssay's limit for detecting *KRAS*/*BRAF* mutations was exemplified using 0.5 ng of tumor cell line DNA mixed with 50 ng of wild-type DNA as PCR templates. Suppression of wild-type amplification by PNA clamping using 50 ng of wild-type DNA was found to be complete, whereas *KRAS*/*BRAF* mutations contained in cell line DNA were unambiguously identified, demonstrating an analytical sensitivity of 1% for the StripAssay (Fig. 1). Assay sensitivity was unaltered when DNA input ranged from 10 to 50 ng. For some *KRAS*-negative samples, however, nonspecific background signal could be observed when DNA input exceeded 50 ng (data not shown).

3.2. Detection of KRAS/BRAF mutations in clinical samples

All 60 FFPE CRC samples could be analysed with the *KRAS-BRAF* StripAssay, thus identifying 28 (47%) and 2 (3%) samples to harbour mutant *KRAS* and *BRAF*, respectively (Table 1). Of 28 *KRAS* mutant samples, 23 (82%) and 5 (18%) were positive for codon 12 and codon 13 mutations, respectively. Of note, 2 samples contained 2 codon 12 mutations. Ten (36%) of 28 *KRAS* mutant samples were Asp12, followed by 8 (29%) tumors containing the Val12 mutation. Less frequent *KRAS* mutations were Asp13 (4 samples), Ser12 (4 samples), Cys 12 (3 samples), and Cys13 (1 sample).

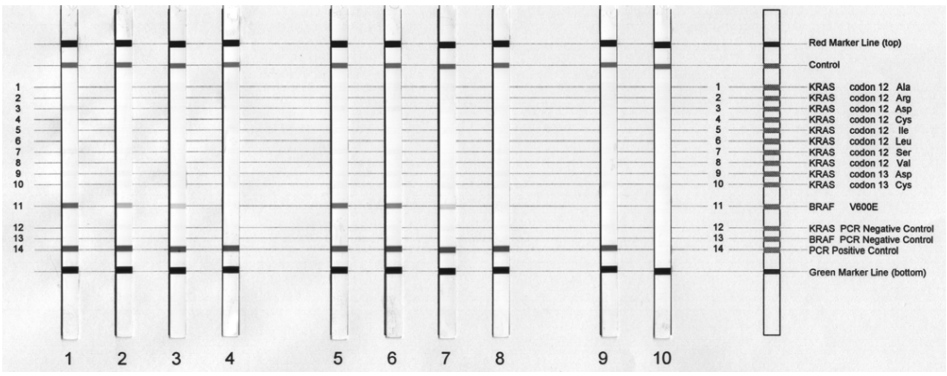

Fig. 2. Detection of *BRAF* V600E in dilutions of DNA extracted from 2 mutant FFPE samples (DNA samples 23 and 29). Test strips obtained after reverse-hybridization and enzymatic color development are shown. The *KRAS-BRAF* StripAssay was performed on a total of 50 ng of sample 23 (strips 1–4) and sample 29 (strips 5–8) mixed with wild-type DNA at 50% (strips 1 and 5), 10% (strips 2 and 6), 1% (strips 3 and 7), 0% (strips 4 and 8), and 50 ng wild-type DNA alone (strip 9). PCR non-template control (strip 10).

Table 1
KRAS/BRAF mutations detected by reverse-hybridization and reference methods

Sample	UICC stage	*KRAS-BRAF* StripAssay	Reference
1	II	*KRAS* Asp12	*KRAS* Asp12[a]
2	III	*KRAS* Cys12	*KRAS* Cys12[a]
3	II	*KRAS* Asp12	*KRAS* Asp12[a]
4	II	*KRAS* Asp13	*KRAS* Asp13[a]
5	I	*KRAS* Asp13	*KRAS* Asp13[a]
6	I	*KRAS* Asp12	*KRAS* Asp12[a]
7	II	*KRAS* Asp12	*KRAS* Asp12[a]
8	IV	*KRAS* Asp12	*KRAS* Asp12[a]
9	III	*KRAS* Val12	*KRAS* Val12[a]
10	III	*KRAS* Cys12	*KRAS* Cys12[a]
11	I	*KRAS* Asp13	*KRAS* Asp13[a]
12	III	*KRAS* Ser12	*KRAS* Ser12[a]
13	III	*KRAS* Cys13	*KRAS* Cys13[a]
14	IV	*KRAS* Asp13	*KRAS* Asp13[a]
15	I	*KRAS* Asp12	*KRAS* Asp12[a]
16	IV	*KRAS* Val12	*KRAS* Val12[a]
17	IV	*KRAS* Val12	*KRAS* Val12[a]
18	I	*KRAS* Val12	*KRAS* Val12[a]
19	III	*KRAS* Val12	*KRAS* Val12[a]
20	III	*KRAS* Val12	*KRAS* Val12[a]
21	IV	*KRAS* Asp12/Ser12	*KRAS* Asp12/Ser12[a]
22	II	*KRAS* Asp12/Ser12	*KRAS* Asp12/Ser12[a]
23	II	*BRAF* V600E	*BRAF* V600E[b]
24	IV	*KRAS* Ser12	*KRAS* Ser12[a]
25	I	*KRAS* Asp12	*KRAS* Asp12[a]
26	III	*KRAS* Cys12	*KRAS* Cys12[a]
27	IV	*KRAS* Val12	*KRAS* Val12[a]
28	I	*KRAS* Val12	*KRAS* Val12[a]
29	III	*BRAF* V600E	*BRAF* V600E[b]
30	I	*KRAS* Asp12	*KRAS* Asp12[a]

[a]DNA sequencing; [b]HRM.

All *KRAS* mutations were verified by direct PCR sequencing (Table 1). Moreover, test strip hybridization identified 2/60 (3%) samples to carry the *BRAF* V600E mutation. The same samples had previously been found to be mutated by HRM analysis (Table 1).

To investigate whether the *KRAS*/*BRAF* StripAssay is capable of detecting mutations with the same sensitivity in clinical specimens (ie, an analytical sensitivity of 1% was determined using cell line dilutions), we prepared serial dilutions of FFPE-extracted genomic DNA obtained from those 2 samples that had been identified to contain *BRAF* V600E. Figure 2 shows that test strip hybridization again was able to detect mutant DNA present at a 1% level.

4. Discussion

Enormous progress occurred in our understanding of the role of the RAS-RAF-MEK-ERK pathway of both inherited genetic variations and somatic genetic changes arising during cancer development [18,19]. This has prepared the way to an individualized therapeutic approach in the medical treatment of a variety of human cancers, such as CRC, ovarian cancer, lung cancer, melanoma and PTC [20–26]. The selection of appropriate candidates for these therapies is of great clinical importance, especially to spare patients from inappropriate treatment. Considering the fact that *KRAS* mutations only account for approximately 35–45%, and *BRAF* V600E adds another 10–18% of non-responders to targeted therapy in CRC, combined testing for *KRAS*/*BRAF* mutations is useful to select appropriate patients for anti-EGFR therapies [20].

In this study, we evaluated a non-quantitative reverse-hybridization assay (*KRAS-BRAF* StripAssay) designed for the simultaneous detection of 10 mutations in codons 12 and 13 of the *KRAS* gene and *BRAF* mutation V600E. First, dilution experiments us-

ing different amounts of tumor cell line DNA were performed to determine the StripAssay's limit for detecting *KRAS*/*BRAF* mutations. Subsequently, the *KRAS-BRAF* StripAssay was used to screen 60 FFPE primary tumor samples previously analyzed for the presence of *BRAF* V600E by HRM.

Test strip hybridization was able to detect *KRAS*/*BRAF* mutations present in 0.5 ng mutant cell line DNA diluted with 50 ng wild-type DNA, thus demonstrating an analytical sensitivity of 1% for the *KRAS-BRAF* StripAssay. Assay sensitivity was maintained for total DNA input amounts between 10 and 50 ng. Too much input DNA (i.e. $>$ 50 ng), however, was associated with a higher chance of background signal in *KRAS*-negative samples most probably caused by aberrant coamplification of wild-type sequence and non-specific binding of resulting PCR products to mutant-specific capture probes.

Of 60 FFPE CRC samples, test strip hybridization identified 28 (47%) and 2 (3%) samples to be positive for *KRAS* and *BRAF* mutations, respectively. *BRAF* V600E results concurred with those obtained by HRM analysis and DNA sequencing. Additionally, *KRAS* and *BRAF* mutations were found to be mutually exclusive. These findings are consistent with published studies that detected mutant *KRAS* and *BRAF* in 20% to 45% [27,28], and 2% to 10% of CRC cases [29], respectively.

Using a cell line dilution model, the *BRAF* HRM assay demonstrated an analytical sensitivity of 1% mutant sequence in a background of wild-type DNA, however, sensitivity fell to 20% when the assay was performed on FFPE samples at a DNA template concentration of 2 ng/μL [16]. In contrast, *KRAS-BRAF* StripAssay sensitivity remained unaltered using the same FFPE sample at the same DNA template concentration. This result corroborates other studies that found test strip hybridization suitable for the sensitive detection of *KRAS* mutations in FFPE DNA samples derived from ovarian and colorectal tissue [13–15]. It seems worth mentioning that high sensitivity *KRAS/BRAF* analysis might detect subclonal mutations in samples with tumor cell contents $>$ 30%, and little is known about the clinical significance of such mutations. However, recent research demonstrated, that *KRAS* mutations present in minor clones may have an important impact on EGFR-directed therapies. This was shown by studying 83 lung cancer patients treated with tyrosine kinase inhibitors using direct an mutant-enriched sequencing for *KRAS* analysis [30].

Various PCR-based methodologies, such as enriched PCR-restriction fragment length polymorphism (PCR-RFLP) [31,32], denaturing high performance liquid chromatography (DHPLC) [33], amplification refractory mutation system (ARMS) [34], particle flow cytometry [35], DNA sequencing [36], HRM [16,37–39], and test strip hybridization [13–15] have been successfully applied to *KRAS*/*BRAF* mutational screening in a variety of cancerous tissues.

HRM is a rapid, closed-tube, post-PCR method that allows high-throughput analysis of mutations, polymorphisms and methylation patterns. By studying the thermal denaturation of double-stranded DNA in the presence of a saturating dye, the most important HRM application is gene scanning, however, genotyping protocols using unlabeled probes have also been reported recently [39]. Although HRM analysis does not require post-PCR sample handling, subsequent DNA sequencing is neccessary to identify specific mutations when genotyping is performed with saturation dye only. Unlabeled probe genotyping supersedes the need for DNA sequencing, however, individual reactions are necessary for each mutation to be analysed thereby increasing cost as well as DNA input. The *KRAS-BRAF* StripAssay allows simultaneous detection of 10 common *KRAS* mutations and *BRAF* mutation V600E with an analytical senitivity of 1%, whereas the limit of HRM for detecting *KRAS*/*BRAF* mutations ranges from 1% to 6% mutant sequence in a background of normal DNA [16,37]. In its current version, however, the *KRAS-BRAF* StripAssay will miss other V600 *BRAF* mutations (e.g. V600D, V600K) that may be present in cancerous tissue. Test strip hybridization requires standard laboratory equipment only, and up to 20 samples may be processed manually in less than 6 hours excluding DNA isolation. For higher sample throughput, test strip processing may be automated to a larger extend by using commercially available equipment [13].

The knowledge of specific genotypes for the assessment of prognosis and therapeutic decisions is rapidly increasing and changing. In this context, the *KRAS-BRAF* StripAssay offers a robust tool for the sensitive detection of *KRAS*/*BRAF* mutations in FFPE-extracted DNA.

Acknowledgments

The authors wish to thank Bettina Rauscher (ViennaLab) for excellent technical assistance.

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