RAS Signaling in Colorectal Carcinomas through Alterations of RAS, RAF, NF1, and/or RASSF1A[1]

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Abstract

More than half of all colorectal carcinomas are known to exhibit an activated mitogen-activated protein kinase pathway. The *NF1* gene, a negative regulator of KRAS, has not previously been examined in a series of colorectal cancer. In the present study, primary colorectal carcinomas stratified according to microsatellite instability status were analyzed. The whole coding region of *NF1* was analyzed for mutations using denaturing high-performance liquid chromatography and sequencing, and the copy number alterations of *NF1* were examined using multiple ligation-dependent probe amplification and real-time polymerase chain reaction. The mutational hot spots in *KRAS* and *BRAF* were sequenced, and promoter hypermethylation status of *RASSF1A* was assessed with a methylation-specific polymerase chain reaction. One sample had two missense mutations in *NF1*, whereas nine additional tumors had intronic mutations likely to affect exon splicing. Interestingly, 8 of these 10 tumors were microsatellite-unstable. Four other tumors showed a duplication of *NF1*. Mutations in *KRAS* and *BRAF* were mutually exclusive and were present at 40% and 22%, respectively. *RASSF1A* was hypermethylated in 31% of the samples. We show that the RAS signaling network is extensively dysregulated in colorectal carcinomas, because more than 70% of the tumors had an alteration in one or more of the four examined components.

Neoplasia (2008) 10, 680–686

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths in the western world today, and at least 50% of CRCs are thought to have a dysregulation of the RAS-RAF-MEK-ERK pathway, also known as the mitogen-activated protein kinase (MAPK) pathway [1]. When activated, this pathway leads to increased proliferation and reduced apoptosis, two of six crucial abilities of a cancer cell, as described by Hanahan and Weinberg [2]. There are several components in this pathway, which, theoretically, could be affected in cancer, and some are known mutational targets in cancer such as *KRAS* and *BRAF*. *KRAS* has been widely established as an important

oncogene since its first mutational report in 1984 [3], and it is now known that it is mutated in 21% of all human sporadic cancers, including one third of CRCs [1].[2] *BRAF* was shown to be a mutational target in cancer 5 years ago [4], and 20% of all human cancers harbors a mutation, including an estimated 13% of colorectal carcinomas.[2] Another potential target of this pathway is the *NF1* gene, which encodes neurofibromatosis type 1, a GTPase-activating protein (GAP), governing hydrolysis of KRAS-GTP to KRAS-GDP [5], thereby functioning as a negative regulator of KRAS signaling. The *NF1* gene is approximately 280 kb in size and maps to chromosome 17q11.2. It contains 61 exons, with an 11- to 13-kb transcript and an open reading frame coding for 2818 amino acids. There are two catalytical domains in NF1, which are important for its function, namely, the cAMP/PKA domain comprising exons 11 to 17 and the RAS-GRD (RAS GAP-related domain) domain comprising exons 21 to 27a [6–8]. Neurofibromatosis type 1, a dominant disorder, is caused by mutations in *NF1*, but somatic mutations in this gene can also contribute to tumorigenesis. Since the first mutation report of the gene in 1992 showing that one colorectal tumor (of 22) was mutated in *NF1* [9], it has been speculated to play a role in colorectal tumorigenesis. However, due to the large size of the gene and the fact that there are no mutational hot spots, mutation analysis of *NF1* in tumors has been very scarce. *RASSF1* (Ras association domain family 1) gene maps at chromosome 3p21.3, and its isoform A (*RASSF1A*) has been found hypermethylated in 40% of lung tumors [10] and in a large variety of human cancers, including CRC [11,12]. As implied by its designation, RASSF1A is thought to interact with KRAS through a Ras association domain that alters its effects. RASSF1A has several effects, including promotion of apoptosis, cell cycle arrest, and maintenance of genomic stability, abilities typical of tumor suppressor genes. Some of these effects refer to the negative regulation of KRAS [13]. Its association to, and its effect on, KRAS is still not solved, although increasing evidence points to a direct binding between RASSF1A and farnesylated KRAS (reviewed in the study of Donninger et al. [11]). The *KRAS* and *BRAF* mutation status together with the alteration of other upstream components affecting the RAS signaling have been reported for other cancers [14], but only two previous studies have examined alterations in *KRAS*, *BRAF*, and *RASSF1A* in the same series of colorectal neoplasms [15,16], and independent of cancer type, no previous study has included a detailed analyses of the *NF1* gene.

To provide further insight into the role of MAP kinase signaling in CRC, we carried out the first comprehensive mutation analyses of the *NF1* gene in colorectal carcinomas in comparison with alterations of *BRAF*, *KRAS*, and *RASSF1A* in a sample series selected to include a comparable number of samples with and without the microsatellite instability phenotype.

Materials and Methods

Tissue Specimen

Sixty-five sporadic colorectal carcinomas from 64 patients with a mean age of 70 years (range 33–92 years), and an equal distribution of male–female were included in the present study. Twenty-nine samples displayed microsatellite instability (MSI), whereas 36 were microsatellite-stable (MSS). All tumors were nonfamilial as assessed by written questionnaires and cross check with the Norwegian Cancer Registry [17]. The colon, including the rectum, was divided into proximal and distal sections: the proximal, or right side, spans from cecum to two thirds of the way across transversum; the distal, or left side, comprises the last third of the transversum, sigmoideum, and the rectum. Of the 65 samples, 23 were located in the proximal colon and 42 were located in the distal colon. The carcinomas are from a prospective series collected from seven hospitals in the Southeast region of Norway during 1987–1989 and contain, on average, 84% tumor cells [18]. The tumors have been selected to achieve a consistently higher number of MSI-positive tumors compared to the normal distribution (15%). By stratifying the samples according to the MSI status, we ensured that any results associated with the MSI or MSS group would be detected.

NF1 *Mutation Screening — DNA Amplification and Denaturing High-Performance Liquid Chromatography Analysis*

Twenty-four representative CRC samples were analyzed for mutations in the *NF1* gene. These samples were selected to resemble the remaining series with regards to sex, age, tumor location, MSI status, and *KRAS* and *BRAF* mutation status. The 61 *NF1* gene exons were amplified in 61 polymerase chain reaction (PCR) fragments of 172 to 579 bp. The primers were generally positioned approximately 50 to 60 bp from the intron–exon boundary to allow the detection of splicing defects while minimizing intronic polymorphisms. In total, 19,843 bases were screened per sample to obtain the final mutation status. The dHPLC was carried out as previously published [19], with minor alterations in the PCR protocol and denaturing high-performance liquid chromatography (dHPLC) methods. For details concerning the dHPLC, please refer to Table W1.

In short, the initial PCR was carried out in 25 µl of reaction volumes and was cooled at room temperature for 60 minutes to yield heteroduplex formation. The identification of somatic *NF1* gene mutations was carried out with dHPLC on a 3500HT WAVE DNA fragment analysis system (Transgenomic, Crewe, UK) equipped with a DNASep column (Transgenomic). Polymerase chain reaction products were examined through a 5% linear acetonitrile gradient for heteroduplexes with a separation flow rate of 1.5 ml/min. Commercially available WAVE Optimized Buffers (A, B, and D; Transgenomic) and Syringe Solution (Transgenomic) were used to provide highly reproducible retention times with WAVE System instrumentation. Resolution temperatures and starting concentrations of buffer B for dHPLC analysis are reported in Table W1.

Sequencing

For each dHPLC abnormal elution profile, genomic DNA was reamplified with dHPLC primers and directly sequenced in both directions on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were analyzed and compared with the mRNA reference sequence and with the chromosome 17 genomic contig reference sequence (NM_000267). The first base (position +1) of the initiator methionine is taken as the start of the cDNA. All missense and splicing mutations detected were absent on 200 control chromosomes belonging to the unaffected subjects.

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[2]Sanger Institute — Catalogue of Somatic Mutations in Cancer (COSMIC) Web site.

KRAS and BRAF Mutation Screening

The mutational hot spots of *KRAS* (exons 2 and 3) and *BRAF* (exons 11 and 15) were directly sequenced in both directions for all samples (*n* = 65) on an ABI PRISM 377 DNA Sequencer (Applied Biosystems) and an ABI PRISM 3730 DNA Sequencer (Applied Biosystems). All nucleotide numbers are based on the cDNA reference sequence (*BRAF*, GenBank Accession No. NM_004333; *KRAS*, GenBank Accession No. NM_004985). For primer details please see Table W1.

Methylation-Specific PCR of RASSF1A

Methylation-specific PCR (MSP) of *RASSF1A* were performed with published primers [20]. Polymerase chain reaction conditions were as follows: denaturation and enzyme activation at 95°C for 15 minutes; 35 cycles of 30 seconds of denaturation at 95°C, 30 seconds of annealing at 62°C, and 30 seconds of elongation at 72°C; final extension at 72°C for 7 minutes.

Human placental DNA (Sigma Chemical Co., St. Louis, MO) treated *in vitro* with *Sss*I methyltransferase (New England Biolabs Inc., Beverly, MA) was used as a positive control for MSP of methylated alleles, whereas DNA from normal lymphocytes was used as a control for unmethylated alleles. The PCR products were separated using a 2% agarose gel before individual visual scoring by two people. Methylated samples with intensity equal to, or higher than, the positive control were considered to be hypermethylated.

Multiple Ligation-Dependent Probe Amplification Analysis

Screening for *NF1* single- and multiexon deletions was carried out in 24 of the colorectal carcinomas using the SALSA P081/082 NF1 (version 04, 05-02-2005) multiple ligation-dependent probe amplification (MLPA) assay (MRC Holland, Amsterdam, The Netherlands), as instructed by the manufacturer and previously reported [21]. In brief, two probes in each exon were hybridized to the individual tumor DNA, followed by a ligation of the nick between the probes, and PCR amplification with 6-FAM–labeled universal primers. The amplified product was analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and the results were exported to Coffalyzer v.5 Software (MRC Holland). As controls, and in each experiment, we used five normal blood samples taken from healthy individuals who do not show NF1 phenotypic traits as determined by clinical evaluation. Furthermore, these controls are verified to have an unaltered *NF1*, both at the nucleotide and at the copy number level. A ratio of ~1 should be obtained if both alleles are present. A reduction or increase in the peak area values to <0.7 or >1.3 was considered an indication of a deletion or a gain, respectively. DNA samples showing a reduction or increase in the MLPA peak area according to the chosen threshold values were reanalyzed by MLPA, and only the samples showing consistent results between the two experiments were scored as deleted or gained.

Real-Time Polymerase Chain Reaction

The gene gains identified by MLPA were also confirmed with a TaqMan Real-Time PCR experiment using an ABI 7000 Sequence Detection System (Applied Biosystems). Two TaqMan probes mapping in *NF1* exons 25 and 28, respectively, were designed by File Builder 3.1 software (Applied Biosystems). These were amplified separately together with the endogenous control (RNaseP) in 96-well fast plates following the recommended protocol (Applied Biosystems). All samples were analyzed in parallel, and the mean value was used for

data analysis. In cases where *N*-fold was below the maximum *N*-fold copy number observed among the nondeleted DNA used as negative controls, it was accepted that the test sample harbored one copy of the target gene. In cases where *N*-fold resulted above the minimum *N*-fold copy number observed among the nondeleted DNA, it was accepted that the test sample harbored two or more copies of the target gene.

Statistics

For this study, 2 × 2 contingency tables were analyzed using Fisher's exact test, whereas 3 × 2 tables were analyzed by the Pearson chi-square test. An independent *t* test was performed when comparing continuous normally distributed data between two groups. All *P* values were derived from statistical tests using the SPSS Version 15.0 software (SPSS, Chicago, IL), and considered statistically significant at $P \leq .05$.

Results

NF1

One of the 24 carcinomas contained two missense mutations (D1302Y and V2577G), the first located within the RAS-GRD domain. *In silico* protein modeling showed that D1302Y has lost an exposed negative charge, which may be important in protein folding and in binding to other proteins. The V2577G most likely has no effect on the neurofibromin function. Additional nine tumors displayed intronic mutations in the range of 4 to 57 bases away from the intron–exon boundary (Table W2).

Using MLPA, we found that another 4 (17%) of 24 samples had a gain of parts or of the whole gene, also confirmed with real-time analysis (Table W2).

Comparison of the molecular results with clinicopathologic data showed that 8 of 10 samples with exonic or intronic alterations in *NF1* occurred in MSI-positive tumors (*P* = .047), whereas 3 of 4 duplications occurred in MSS tumors.

KRAS

Direct sequencing of exons 2 and 3 of *KRAS* revealed that 26 (40%) of 65 tumors harbored a mutation (Table W2). All but two mutations were missense mutations and occurred in codon 12, 13, or 61. These two were a 3-bp insertion (TGG) in exon 2 (c.49insTGG) and a 3-bp deletion in exon 3, codons 62 to 63 (c.184_189delGAG; Table W2). Furthermore, two of the tumors had two *KRAS* mutations each. One displayed both G12A and V14I mutations, and the second had both G12D and G13D.

Mutations in *KRAS* were seemingly more often present in MSS tumors than in MSI tumors, 69% *versus* 46% (*P* = .08).

BRAF

Mutational analysis of *BRAF* gene revealed that 14 (22%) of 64 samples harbored a mutation. Eleven of these were the typical V600E mutation; the remaining three were D594G, L597Q, and G1406C (Table W2).

Mutations in *BRAF* were strongly associated to MSI, female gender, and proximal location (*P* = .006, *P* = .015, and *P* = .025, respectively). Figure 1 illustrates the individual localization of each mutation in *KRAS*, *BRAF*, and *NF1*.

Figure 1. Site distribution of mutations within each gene. The mutations for the respective gene are placed according to their sequence position. In (a) and (b), only the exons in blue have been analyzed. In (c), all exons are analyzed, and the exons in orange indicate those that are only expressed in isoforms. To the right, representative sequencing results of mutant samples are presented.

RASSF1A

By MSP analysis, we found that 18 (31%) of 59 samples were hypermethylated in the promoter of *RASSF1A*. Methylation of the gene was more frequent in the distally located tumors (P = .041), but was not overlapping with the MSS phenotype. In eight tumors, hypermethylation of *RASSF1A* was the only observed alteration among the four genes analyzed here. We found no covariance between *RASSF1A* methylation and mutation status of either of the analyzed genes.

Dysregulation of RAS Signaling

When looking at concurrent mutations in individual tumors, we found that *KRAS* and *BRAF* were mutually exclusive because all *BRAF* mutations occurred in wild type *KRAS* tumors and *vice versa* (P < .0001). The sample with *NF1* missense mutations was MSI-positive, proximally located, and harbored a *BRAF* mutation. When including the intronic mutations in the number of *NF1* mutations, six of eight *BRAF* mutations occurred in *NF1*-mutated samples

(P = .03), overlapping with the MSI. Three of the four duplications found in the *NF1* locus occurred in tumors with wild type *BRAF* and *KRAS*. The remaining tumor had both a *KRAS* mutation and duplication.

The occurrence of *RASSF1A* hypermethylation in the presence of other mutations did not show any trends toward coexistence or mutually exclusive nature.

Taken together, we found that 74% (48/65) of the tumors had an overactive RAS signaling pathway due to change of at least one of the four analyzed components (one alteration in 37/48, two alterations in 10/48, and three alterations in 1/48). For the 24 samples submitted for complete analyses, the number of samples with at least one alteration was 19 (79%): one alteration was seen in 14 samples, two alterations were seen in 4 samples, and three alterations were seen in 1 sample. All samples and alterations are summarized in Figure 2 and Table W2.

Discussion

This is the first report with an extensive analysis of the role of *NF1* mutations in colorectal tumorigenesis. Previous mutation studies have only looked at a small number of samples, usually in a limited part of the gene, in the RAS–GAP domain. The initial mutational report on *NF1* showed that 1 of 22 colorectal adenocarcinomas harbored a mutation in the RAS–GAP domain using single-strand conformation polymorphism [9]. Another study of 10 colorectal cell lines and 4 sporadic tumors using protein truncation test disclosed mutations in the *NF1* coding region in four MSI cell lines (40%) and one MSI tumor (25%). Two of the cell lines had in fact two mutations each [22]. A recent study examined five hereditary nonpolyposis CRC patients for mutations in five exons and found a mutation in one (20%) of the patients who had a homozygous germline mutation of *MLH1* [23]. Moreover, loss of heterozygosity (LOH) at loci within the *NF1* gene have been shown in primary colorectal tumors (range, 14–57%) [24,25]. One of these studies also used real-time expression studies of *NF1* in 55 of the carcinomas and found an

increased expression among tumors compared with normal colon tissue. In the COSMIC database [26], 79 carcinomas of the colorectum were apparently included among the *NF1* data, yielding a mutation frequency of 11%. However, seven of nine mutations reported were from one study including seven cell lines, leaving only two of the mutations occurring in sporadic primary tumors.

In this study, we found the *NF1* mutation profile to be in contrast to published germline mutation profile of NF1 patients[3] as well as to the somatic mutation profiles of malignant peripheral nerve sheath tumor taken from patients with and without the NF1 disease [27,28] (Bottillo I et al., unpublished observations). Furthermore, the median age of the patients included in the present CRC series is old, suggesting that potential NF1 carriers among them should have shown a debut of an NF1-associated cancer type. As no typical NF1-associated tumors are recorded, based on written questionnaires and confirmation of cancer diagnoses from the Norwegian Cancer Registry [17], it further support that the reported mutations are somatic. The observed intronic mutations prevailing among the colorectal tumors could be involved in alternative splicing but this remains to be elucidated. Four of the nine intronic mutations were indels of one or two bases in microsatellites and reflect replication slippage (which often occurs in such repetitive stretches of bases) left unrepaired by the defective DNA mismatch repair system [29]. No such indels were found in *KRAS* or *BRAF*.

Multiple ligation-dependent probe amplification results showed that 17% of the analyzed samples had gained parts of or whole of the *NF1* gene. This is not in accordance with the expectations of a tumor suppressor gene involved in tumorigenesis. A duplication of *NF1* could lead to a stronger negative regulation of KRAS, with subsequent stronger control of proliferation and differentiation. The duplications may arise as a consequence of the chromosomal instability present in three of the four tumors, which yield a wide range of gains and losses of whole or parts of chromosomes. As reported by Ĉaĉev et al. [30], colorectal tumors show a significant increase of *NF1* mRNA expression compared with corresponding normal tissue. They also showed that the expression of *NF1* isoform I (lacking exon 23a, located in the middle of the RAS-GRD domain) was significantly higher in tumor compared with normal tissue [30]. As of this, the present findings are in agreement with those of the study by Ĉaĉev et al. [30].

We also found a 40% mutation frequency of *KRAS*, which is within the expected range [26]. A mutated *KRAS* (in codons 12, 13, and 61) hinders the hydrolysis of GTP to GDP, and will keep KRAS in a constitutively active state, leading to phosphorylation of downstream effectors such as BRAF [31]. *BRAF* mutations are known to be strongly associated with MSI and CpG island methylator phenotype [32,33] and are found very often mutated in sessile-serrated adenomas, lesions often considered as a precursor of MSI-H tumors [34–38]. We found *BRAF* mutation in 22% of the samples, a higher frequency than in the mutation databases [26]. This reflects a bias due to the enrichment of MSI tumors in the present series. In one study, 71% of the MSI tumors had a V600E mutation in *BRAF*, as opposed to 7% in the chromosomal-unstable tumors [39], a figure comparable with the present series, as 18 (62%) of 29 of MSI tumors had *BRAF* mutations. The most common *BRAF* mutation, V600E, just as the common *KRAS* mutations, will lead to a constitutively active protein, as the activation loop of the protein is changed [31].

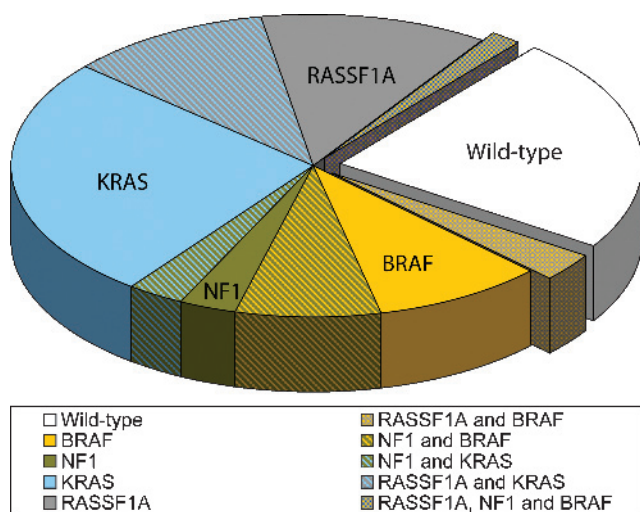**Figure 2.** Alterations across the sample series. The pie chart indicates the four analyzed components and the percentage of tumors which showed alterations among these. Clockwise from the wild type pie, we see alterations in *RASSF1A* and *BRAF; BRAF; BRAF* and *NF1; NF1; NF1* and *KRAS; KRAS; KRAS* and *RASSF1A; RASSF1A; RASSF1A, NF1* and *BRAF*.

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[3]NF1 International Mutation Database (http://www.nfmutation.org).

Some studies indicate an indirect interaction between RASSF1A and KRAS through RASSF5 (previously annotated NORE1A) [12], whereas others argue for a direct binding between RASSF1A and activated, farnesylated, KRAS [11]. Previous studies have also included *RASSF1A* when analyzing the impact of *KRAS* and *BRAF* mutations in colorectal tumorigenesis [13,15,16,40], and none of them found any co-occurrence between *RASSF1A* methylation and *BRAF* or *KRAS* mutation, in line with the present finding.

When adding the data of the fourth component, *NF1*, of the RAS signaling pathway, we found that more than 70% of the samples had a hyperactive RAS signaling. As the effect of RASSF1A on RAS signaling is still unclear, the eight samples with the sole alteration being hypermethylation may not be important for an overactive RAS signaling pathway. When we exclude the *RASSF1A* data from the combined analysis, 62% (40/65) of the samples had an overactive RAS signaling network, all due to *KRAS* or *BRAF* mutations, as the sample with the *NF1* missense mutations overlapped with *BRAF* mutation. If we include the *NF1* changes potentially affecting the splicing, 77% of the tumors have a dysregulation of the RAS signaling pathway.

In conclusion, we show that the RAS signaling network is extensively dysregulated in colorectal carcinomas as more than 70% of the tumors have an alteration in one or more of the four components.

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Table W1. Primer Sequences and dHPLC Conditions.

Exon	Primer FW	Primer RV	T_m PCR (°C)	Ampl. Size (bp)	tdHPLC	% B
1	5′-CTCCACAGACCCTCTCCTTG-3′	5′-GGACAGAGTAGGTGAGGGGA-3′	58	242	64–68	57–54
2	5′-AAACGTCATGATTTTCAATGGC-3′	5′-GGGGAATTTGCTTTCTTTTCTT-3′	58	281	55.5	56.6
3	5′-TTTCACTTTTCAGATGTGTGTTG-3′	5′-CTTTGTGAATTTGATCTTGAG-3′	58	210	55.5	54.8
4a	5′-GTTTGAAAATTTTCATAATAGAAA-3′	5′-CTCACAGCAGCTTTGACCTCC-3′	58	417	51–57	61–57
4b	5′-CAAGTGGTCCTCCTGCCTT-3′	5′-GTCAAAAACTAGTATCATGAATG-3′	58	283	55	55
4c	5′-TTTCCTAGCAGACAACTATCGA-3′	5′-ATTTGCTGTTGTTAGCATCCT-3′	62	308	54.5	57.5
5	5′-GAAGGAAGTTAGAAGTTTGTGACA-3′	5′-ATGGCTGGTAAGGATACGATTG-3′	62	172	54	51
6	5′-CATGTTTATCTTTTAAAAATGTTG-3′	5′-ATGTGAAGCAGTTTATTTTACTCAA-3′	62	332	54.5–56	58.6–56.6
7	5′-ATTTGCTATAATATTAGCTACATCTGG-3′	5′-GTTGATAAGTTCATAGGACTTGCTTT-3′	62	385	53–56.5	58–54.3
8	5′-GGATTTTACTGCCATTTGTGTG-3′	5′-TATCTAACTATATTTACTGATGCTGTTA-3′	58	276	56	56
9	5′-GCTGTTCTTTTTGGCTTC-3′	5′-CCAAAAGGTATTGCTAAATTAC-3′	58	183	54.5–56	52.2–49.7
9br	5′-GCTTAAAATTTGTATACAATAAAC-3′	5′-CCTGGAGTGGTGCTTCATGCAT-3′	58	193	55–60	52–48
10a	5′-CTACAGTGATAAACAGAGCAT-3′	5′-ATTCCTGCTGCTTTGGTT-3′	62	292	55–58	58–55
10a2	5′-CATTTTTTTGGTGTTTATGTATAGCAAG-3′	5′-GTGTATAGTTACCATTATAGTCACATC-3′	62	252	54–57–60.5	57–54–50
10b	5′-ATTATCCTGAGTCTTATGTC-3′	5′-TCTCAAAATTATCACACTAAGTTA-3′	58	229	54–57	56.2–51.2
10c	5′-ACCCTTTAGCAGTCACTGTC-3′	5′-CTGTGAGTAACAGGTAGATG-3′	58	307	54–59	59–54.4
11	5′-GAAAGAGCTCAATTTCTTAGC-3′	5′-CACTTCCAAAGGTTTTATGGT-3′	58	307	52–55	58–55
12a	5′-TGTATTCATTATGGGAGAATGCC-3′	5′-TGGAAGAATATTTGGAATGGTAAT-3′	58	269	54–56	56–54
12b	5′-GAGGTTTTTTAGGAGAGTCTC-3′	5′-ATGTGCTCTGTTTGTTTTCTG-3′	58	315	54–57.5	58–53.4
13	5′-CACAGTTTATTGCATTGTTAG-3′	5′-CTGCCTCAAAGCACATGG-3′	62	380	57–61	59–54
14	5′-GCTCTTCCTACTCCTTTTGG-3′	5′-TATGCCCTTAGCAACAGAAA-3′	58	191	60	54
15	5′-ACTTGGCTGTAGCTGATTGA-3′	5′-TCAAGAGTCGCTCAGTAAAGT-3′	62	247	57	57
16	5′-CATTTTTTGTACTTTTGTCATGG-3′	5′-CTCTTATTTTTCACCTTTCTC-3′	58	579	55–58	63–60
17	5′-ATTTGGCTCTATGCCTGTGG-3′	5′-ACTGCACACAAACTAGGGTG-3′	58	385	55.5	58.8
18	5′-AGAAGTTGTGTACGTTCTTTTCT-3′	5′-GCGGTTATTGGTAGAAAGGAG-3′	58	367	53–56	57–54.4
19a	5′-TCATGTCACTTAGGTTATCTGG-3′	5′-CCTTCAAGTATTAGTGGGTTTTA-3′	58	242	55–57.5	58–55.7
19b	5′-TGAGGGGAAGTGAAAGAACT-3′	5′-GCAAAAAGCAAATAAAGCC-3′	58	236	53.5–57.5	56.5–52.5
20	5′-CCACCCTGGCTGATTATCG-3′	5′-GCATGTAAGAGAAGCAAAAATTA-3′	62	402	57–59	59–57
21	5′-AGCAAAAATTACTTCAGCAA-3′	5′-TCAGAGCCAGAAGAAAGATG-3′	58	393	57–59	59–57
22	5′-TGCTACTCTTTAGCTTCCTAC-3′	5′-GGCTGATTGTCTTCTTTTAAGG-3′	58	331	56.5–58	58.6–57
23.1	5′-TTTGTATCATTCATTTTGTGTGTA-3′	5′-CTTTTCACATAGAACCGCTGTTTTTT-3′	58	283	56–57	58.2–57.2
23.2	5′-GGCTTAATGTCTGTATA-3′	5′-GAGATTACCATTATTAATCTAAAGT-3′	58	270	53–59	57–51.3
23a	5′-AGCACAGAAATAGTAGACATGATTGGG-3′	5′-TCTACTAATTCTGGCACAAAATAG-3′	62	446	54.5	60.3
24	5′-TTGAACTCTTTGTTTTCATGTCTT-3′	5′-GATAATCTAGCTATCTTAAATTCC-3′	58	266	53–58	57–52.1
25	5′-AATTTATAGAATGAGGAATG-3′	5′-GTACCTGTTTTACATGAAGTTCCT-3′	54	335	52–54–57	58–56–53.7
26	5′-GCTTTGTCTAATGTCAAGTCA-3′	5′-GATAGTGAACACTCTCCGTTTAA-3′	62	342	56–58	58–54
27a	5′-ATGGTCCTGAGGTCTTTTTG-3′	5′-GCCACCAGGCCACTTGTTAG-3′	62	361	57	59
27b	5′-TTGCTTTTAAAATATTTTTTCATTTTAG-3′	5′-CCCAGTTGACTTAACAGGAATT-3′	58	330	55	55
28	5′-AAAATAAAATTGATTAGTGGCATCTG-3′	5′-AAATGTCACGTAAGGCTGTCG-3′	62	636	55–58	62–60
29	5′-TCTGGAGCCTTTTAGAATTTTATGT-3′	5′-TCAGTTTGATTTGGGGTTTGTTGC-3′	62	460	58–60.5	60–55.5
30	5′-GAAAAAATTTTGGAACTATAAGG-3′	5′-TAACAATTATTCTAAGAGAATTCAAAG-3′	58	322	51–56.5	58–54
31	5′-TTTTTTTCCCCGAATTCTTTATG-3′	5′-CTTCAGAAAGCATGTAGACACTCAC-3′	58	425	55–57	61–59
32	5′-ATCTAGTATTTTTGAGGCCTCAG-3′	5′-CCTTCTGTACTATAGCATATCTG-3′	58	312	53–56	58–55
33	5′-TGCTAAAACTTTGAGTCCCATG-3′	5′-GTGCTCTAACACCAAGTTGC-3′	64	448	56–59	59–53.8
34	5′-TTCTAAATTCAAAATGAAACATGG-3′	5′-AAAAACACTTGCATGGACTG-3′	58	432	51.5–57	60–55
35	5′-GCATGGACTGTGTTATTGGTA-3′	5′-TCTGTGGATCTTTTAATTGCA-3′	58	319	53.5	56.8
36	5′-GCTGGACCAGTGGACAGAAC-3′	5′-GACGTTTAAATTTGAGGTCAATGA-3′	62	389	53–58	57.8–54.3
37	5′-TCCTGAATTCATTCCGAGATT-3′	5′-TCATTTTGGGTATCAGTGTTGAA-3′	58	237	54–56	55.5–53.5
38	5′-AACTGCAGTGTGTTTTGAAAGAG-3′	5′-GAGGTTCCTAGATTACTCAAATTTAG-3′	62	257	57–60	56–53
39	5′-TTGAACACAAAATTAAGTGAGCC-3′	5′-GAAGTAAGTTAGCCCTTATGTCTTAC-3′	62	318	56	55
40	5′-ATTCACATTCACATATGCATGTTTTACCTTC-3′	5′-CTTTGGTTCAAGACACTACAG-3′	62	547	55–56	61.6–61.1
41	5′-GTGCACATTTAACAGGTACTAT-3′	5′-ATCTAGAGATGGCCTAGGAAG-3′	62	373	55.5	58
42	5′-CTTGGAAGGAGCAAACGATGGTTG-3′	5′-CCATGTCAGTGTAGCAAAGTTTTTG-3′	58	356	55–60	56–52.2
43	5′-AGTGTATTCCCATTTATAGACACTG-3′	5′-CATTGAAAATAAGGTGGAGA-3′	58	234	55–57.5	56–52.4
44	5′-GAAGTAACATTGAAATAGTTAGG-3′	5′-TCCAGTCTACTTTTAGGAGGCC-3′	58	271	58.5	55
45	5′-CATGAATAGGATACAGTCTTCTAC-3′	5′-GTTAAATGCTTACCCAGTAATGTG-3′	62	269	57	56
46	5′-CTCATCTCCCTTTAATTTTGGC-3′	5′-TCTGGAGAAGGATGGTTGATG-3′	58	295	54–56.5	57–55.1
47	5′-CTGTTACAATTAAAAGATACCTTG-3′	5′-GTATGCCTGCTTTAAGAACACACA-3′	62	185	55.5	51.4
48	5′-AAGGAAGAAAAATAGTAAATTAAGTCC-3′	5′-GTTTATAGCAAATTTTGCTCCTT-3′	58	423	53–58	61–56.9
48a	5′-ATTCAATAATTAAAACCAGATTCC-3′	5′-CTTTAGGAACTTGTAAAGCCACC-3′	58	327	54	58
49	5′-AGAATGTGTCCCCCGTTGTTAA-3′	5′-TAATGAACCCATCCGGTTTG-3′	58	369	58.5	58.4
KRAS ex2	5′-ACTGGTGGAGTATTTGATA-3′	5′-GTATCAAAGAATGGTCCT-3′	50	—	—	—
KRAS ex3	5′-ATAATAGCCAATCCTAA-3′	5′-ATGGCATTAGCAAAG-3′	53	—	—	—
BRAF ex11	5′-TCATAATGCTTGCTCTGATAGGA-3′	5′-GGCCAAAAATTTAATCAGTGGA-3′	60	—	—	—
BRAF ex15	5′-TCCCTCTCAGGCATAAGGTAA-3′	5′-CGAACAGTGAATATTTCCTTTGAT-3′	58	—	—	—

T_m *PCR*, indicates PCR melting temperature (°C); *Ampl. Size*, amplicon size; *tdHPLC*, range in temperature used with high-performance liquid chromatography; *% B*, starting concentration for buffer B used in dHPLC.

Table W2. Detailed Somatic Events of Four Components in the MAPK Pathway.

Tumor ID	MSI Status	$KRAS^{mut}$	$BRAF^{mut}$	$NF1^{mut}$	MLPA	Real-time	$RASSF1A$
848	MSI	WT	WT	NP	NP		U
854	MSI	c.184-189delGAG	WT	NP	NP		U
884	MSI	WT	V600E	D1302Y/V2577G	WT		U
894	MSI	c.49insTTG	WT	NP	NP		U
910	MSI	WT	WT	c.(3114-50)delTG	WT		U
912	MSI	G13D	WT	NP	NP		U
955	MSI	WT	V600E	NP	NP		U
965	MSI	WT	V600E	NP	NP		U
980	MSI	WT	V600E	WT	WT		U
984	MSI	WT	V600E	WT	WT		U
988	MSI	WT	WT	NP	NP		ND
1022	MSI	WT	WT	NP	NP		U
1044	MSI	WT	V600E	c.480-57C>T	WT		U
1047	MSI	G12A/V14I	WT	WT	WT		M
1066	MSI	WT	WT	WT	WT		M
1117	MSI	WT	WT	NP	NP		U
1132	MSI	G12V	WT	NP	NP		U
1141	MSI	WT	WT	NP	NP		U
1190	MSI	WT	V600E	NP	NP		M
1193	MSI	WT	V600E	c.7395-7C>T	WT		U
1268	MSI	WT	V600E	Ex3+24G>A	WT		U
1273	MSI	WT	V600E	c.(1392+46_+53)delTT	WT		U
1314	MSI	WT	WT	NP	NP		U
1326	MSI	G13D	WT	c.(1392+46_+53)delT	WT		U
1341	MSI	WT	V600E	c.(61-4_-12)delT	WT		M
1349	MSI	WT	WT	NP	NP		M
1363	MSI	G13D	WT	WT	Gain of IVS27b-Ex49	1.66	U
1388A	MSI	WT	WT	NP	NP		M
1388C	MSI	G13D	WT	NP	NP		M
868	MSS	WT	WT	NP	NP		U
886	MSS	G12D	WT	NP	NP		U
887	MSS	G12C	WT	NP	NP		M
896	MSS	WT	WT	WT	Gain of whole gene	1.59	M
904	MSS	WT	WT	NP	NP		U
922	MSS	G12V	WT	NP	NP		U
923	MSS	G13D	WT	NP	NP		U
927	MSS	G12V	WT	NP	NP		M
946	MSS	WT	WT	NP	NP		U
948	MSS	G12R	WT	NP	NP		U
953	MSS	WT	WT	WT	WT		U
966	MSS	Q61L	WT	NP	NP		U
974	MSS	G12A	WT	NP	NP		U
976	MSS	G12D	WT	NP	NP		M
1013	MSS	WT	D594G	NP	NP		U
1024	MSS	G12C	WT	NP	NP		U
1027	MSS	G13D	WT	NP	NP		U
1029	MSS	G12D	WT	NP	NP		U
1046	MSS	WT	WT	NP	NP		U
1060	MSS	WT	WT	NP	NP		M
1069	MSS	WT	WT	NP	NP		U
1103	MSS	WT	WT	WT	Gain of whole gene	1.25	U
1111	MSS	WT	WT	NP	NP		M
1121	MSS	G12A	WT	WT	WT		M
1124	MSS	G12D/G13D	WT	WT	WT		ND
1166	MSS	G13D	WT	WT	WT		M
1167	MSS	WT	WT	WT	Gain of whole gene	1.67	M
1194	MSS	WT	WT	NP	NP		M
1197	MSS	WT	WT	NP	NP		ND
1287	MSS	WT	WT	c.2252-31A>G	WT		ND
1294	MSS	WT	G469R	NP	NP		U
1296	MSS	G13D	WT	WT	WT		U
1340	MSS	WT	WT	WT	WT		ND
1364	MSS	WT	L597Q	NP	NP		M
1369	MSS	G12D	ND	NP	NP		ND
1391	MSS	G12D	WT	c.2252-31A>T	WT		U