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Heterogeneity of Colorectal Cancer (CRC) in reference to KRAS proto-oncogene utilizing WAVE technology

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Abstract

Background—New drugs targeting specific genes required for unregulated growth and metastases have improved survival rates for patients with metastatic colorectal cancer. Resistance to monoclonal antibodies specific for the epidermal growth factor receptor (EGFR) has been attributed to the presence of activating point mutations in the proto-oncogene KRAS. The use of EGFR inhibitor monotherapy in patients that have KRAS wild type has produced response rates of only 10–20%. The molecular basis for clinical resistance remains poorly understood. We propose two possible explanations to explain these low response rates; 1) levels of resistant CRC cells carrying mutated KRAS are below the sensitivity of standard direct sequencing modalities (<5%) or 2) the standard practice of analyzing a single area within a heterogeneous tumor is a practice that can overlook areas with mutated KRAS.

Methods—In a collaborative effort with the surgical and molecular pathology departments, 3 formalin fixed paraffin embedded tissue blocks of human CRC were obtained from the human tissue bank maintained by Lifespan Pathology Department and/or the human tissue bank maintained by the Molecular Pathology Core of the COBRE for Cancer Research Development. The three specimens previously demonstrated KRAS mutations detected by the Applied Biosystems Kit. The Wave system 4500 (High performance ion-pairing liquid chromatography (IP-HPLC)) was utilized to evaluate tissue for presence of KRAS proto-oncogene mutations at codon 12 and 13.

Results—Initially, sensitivity of WAVE technology was compared with direct sequencing by evaluating a dilutional series. WAVE detected mutant alleles at levels of 2.5% compared to 20% performed with standard direct sequencing. Samples from three patients were evaluated by WAVE technology. Eight samples from patient 1 were analyzed. In two of eight samples, no mutations were detected at concentrations as low as 5%. In one sample a mutation was noted by WAVE and not by direct sequencing. All four samples from patient 2 tested positive for Exon 12/13 mutations. Of the seven samples from patient 3, five were positive for Exon 12/13 mutations and two were negative for Exon 12/13 mutations.

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Conclusion—In these studies the analysis of three patients' colorectal cancer tissue were analyzed utilizing the WAVE technology. Results demonstrated a greater degree of sensitivity in mutation detection when compared to standard sequencing. These studies also demonstrated heterogeneity of expression of KRAS mutations between areas of the tissue samples at a genomic level. The low clinical response rates to EGFR inhibition might be explained by the variation in mutation presence, which was dependent upon the region examined. The heterogeneity demonstrated in these studies provides another phenotypic variant that will impact clinical care.

Introduction

In the past decade, the percentage of metastatic colorectal cancer patients surviving for 5 years has approximately doubled; this marked improvement has been attributed to the development new drugs targeting specific genes required for unregulated growth and metastasis. Monoclonal antibodies specific for the epidermal growth factor receptor (EGFR), like Cetuximab, have been effective in previously treated metastatic CRC patients with response rates between 8.8% and 22.9%. [1] Resistance to cetuximab has been attributed to the presence of point mutations in the proto-oncogene KRAS, mutations that can predict resistance to anti-EGFR therapies up to 10 months before radiographic evidence of disease progression. [2, 3] The most frequent mutations in KRAS are point mutations in codons 12 and 13 in 35–45% of CRC and large colon adenomas [4]. KRAS mutations arise early during the development of colorectal carcinogenesis and are maintained throughout CRC development. Both mutations impair intrinsic GTPase activity, which results in constitutive, growth-factor receptor independent activation of down stream events in the KRAS signaling pathway. Clinically, the use of EGFR inhibitors in patients that have KRAS wild-type CRC tumors has shown poor response rates. The molecular basis of resistance to cetuximab in CRC remains poorly understood.

Recent studies have postulated different etiologies for resistance; one hypothesis is that once a somatic mutation is acquired, the genome becomes more susceptible to future mutagenesis; another idea is that with the acquisition of somatic mutations, development of antibodies against therapeutic modalities increases. [5, 6] In order to test these hypotheses, large-scale genetic sequencing efforts are underway which have identified extensive genomic heterogeneity between tumors. It has been postulated that 'intratumor heterogeneity' may have important consequences for personalized medicine relying on targeted therapeutics that are usually chosen based on histopathological analysis of a single tumor biopsy. [7] Clinically, patients exposed to EGFR inhibition will demonstrate 'mixed responses' defined as radiographic response in some areas of tumor burden and potential growth in other areas. This phenomenon suggests intratumor heterogeneity and tumor response to treatment.

Mutation profiling of cancer specimens is limited, by low detection rates resulting from genetic heterogeneity and the presence of normal cells in variable amounts. Current standard molecular methods are based upon Polymerase chain reaction (PCR), which has become an indispensable tool in the diagnosis of disease. [8]

The Wave system 4500 (High performance ion-pairing liquid chromatography (IP-HPLC)) offers an alternative to PCR in providing a broader dynamic range of target DNA quantitation. The WAVE system separates duplexes formed between PCR products and utilizes Surveyor endonuclease, an enzyme that cuts at sites of unpaired nucleotides created by point mutations, polymorphisms (SNPs) and small deletions. The advantages of the Surveyor-WAVE method when compared to PCR include the following: speed and accessibility of analysis; the ability to analyze multiple proteins and multiple tumors at a time; a greater sensitivity in cases of variable expression of a target gene (detection rates as low as 1%)[9]; detection of mutations without prior knowledge of mutation location; and the potential to allow estimation of mutated amplicons.

In this study, colorectal tumor tissue was analyzed utilizing WAVE 4500 mutation analysis system to determine 1) if regions of the tumor, represented by tissue blocks, display heterogeneity in the expression of mutated KRAS and 2) if regions within tissue blocks uniformly display resistant or susceptible phenotypes, a finding that would demonstrate the need for considering phenotypic heterogeneity and choosing targeted therapeutics based on the analysis of multiple tissue blocks.

Methods

In a collaborative effort with the surgical and molecular pathology departments, 3 de-identified tissue blocks of human CRC were obtained from the human tissue bank maintained by Lifespan Pathology Department and/or the human tissue bank maintained by the Molecular Pathology Core of the COBRE for Cancer Research Development. The Wave system 4500 (High performance ion-pairing liquid chromatography (IP-HPLC)) was utilized to evaluate tissue for presence of KRAS proto-oncogene mutations at codon 12 and 13.

All tissue collected for the tissue bank was de-identified and assigned a number as outlined in the protocol reviewed and approved by the Institutional Review Board. If available/applicable, the following Information that was maintained with the tissue: age, sex, tumor type, designated cancer stage, data regarding recurrence of disease and treatment history (limited to prior chemotherapy, radiation or a combination).

Tissue was obtained from three patients who had KRAS mutations detected by direct PCR sequencing as follows; Patient 1 was positive at c.35 G>C (pG12a), patient 2 at C.35 G>A (p612D) and patient 3 at codon 12 GGT>GCT (G12A).

The WAVE 4500 is a high performance liquid chromatography system that relies on ion-pairing chromatography (IP-HPLC) to separate homoduplexes from heteroduplexes formed between PCR products generated with exon specific primers using genomic DNA as template. Heteroduplexes of wild type and mutant exon carry a mismatch that is cleaved by Surveyor endonuclease, an enzyme that cuts at sites of unpaired nucleotides created by point mutations, polymorphisms (SNPs) and small deletions.

Amplification

DNA Samples—Board certified surgical pathologist evaluated hematoxylin and eosin stained slides processed and prepared from FFPE colorectal tumor tissue. The pathologist circled areas that histologically appeared to have densely populated area of carcinoma cells and estimated the percentage of tumor cells within the area. A technician performed macro-dissection of the circled areas to obtain FFPE DNA samples. DNA was extracted using Qiagen QIAamp DNA FFPE Tissue Kit. Samples were deparaffinated and the DNA was extracted into a final volume of 40uL. DNA concentration was determined from 1uL sample DNA using a Thermo Scientific Nanodrop 2000. The DNA samples to be used for amplification were diluted to working concentration using the extraction kit ATE buffer.

PCR—Polymerase chain reaction was performed in a total volume of 25 ul. The reaction mix contained 1 ul of DNA at ~50ng/ul, 2.5 ul of 10X PCR Reaction Buffer, 1.5 or 25 mM MgSO₄, 1.5 uL of 10.0 mM dNTP Mix, .75uL of 10 uM forward primer, .75uL of 10 nM reverse primer, and 1.25 units of Optimase Polymerase. The primers used for this reaction were as follows, Forward: 5'-GTG TGA CAT GTT CTA ATA TAG TCA-3' Reverse: 5'-GTG TAT CAA AGA ATG GTC CTG CAC-3'.

A touchdown PCR method was used for amplification. It consisted of an initial denaturation at 95 C for 5 min; 15 cycles of 95°C for 30sec, 62°C for 30sec and 72°C for 25sec; and 30 cycles of 95°C for 30sec, 55°C for 30sec, and 72°C for 25sec. After a final elongation at 72°C for 2 min, heteroduplexes were formed by denaturing the sample at 95°C for 3min then allowing the DNA to reanneal while cooling the sample to a final temperature of 4°C.

Screening using WAVE Nucleic Acid Fragment Analysis System

Digestion—Four re-annealed PCR products were digested using Transgenomic SURVEYOR nuclease to cleave at mismatch sites in hetero-duplexes composed of wt and mutant KRAS sequences. To each 10.0uL aliquot of re-annealed PCR product, 1.0 uL 0.15mM MgCl₂, 1.0uL enhancer cofactor, 1.0ul Surveyor enhancer, and 2.0uL surveyor nuclease was added. After 20-minute incubation at 42°C, 1.0uL of stop solution was added to each mixture.

Analysis—Detection of point mutations in exons 12 and 13 was performed using the WAVE HS system according to the manufacturers recommended protocol for mutation analysis with surveyor endonuclease. The composition of Buffer A and B were as follows: Buffer A, .1 M TEAA, pH 7.0, and Buffer B, .1M TEAA and 25% acetonitrile. Digested PCR products generated with exon 12/13 specific primers were resolved by ion pairing chromatography on a Transgenomic DNASep column under non-denaturing conditions according to the manufacturer's protocol. In brief, each sample (8uL) was loaded onto the DNA Sep HT column and eluted with a standard gradient at 45°C using a flow rate of 1.2mL/min. Peaks detected by both UV and fluorescence were analyzed using the Navigator Software. For fluorescence detection, reannealed PCR products were incubated with a DNA intercalating fluorescence dye. The sample was passed through the fluorescence detector and the level of fluorescence was recorded over time. This information was processed by the

Navigator Software and records the results in the form of chromatograms. From: <http://www.transgenomic.com/pd/items/nhs-99-4500.asp>

Both the loading buffer and syringe wash solution consisted of 10% acetonitrile for the DNASep® column.

Results were confirmed by direct sequencing using the above-mentioned primers. Briefly, sequencing reactions were performed with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and analyzed on the ABI Prism 3500 Genetic Analyzer (Applied Biosystems). This technology was available through the Molecular Pathology Core of the COBRE for Cancer Research Development.

Results

WAVE results for three patients

Analysis of three patients was conducted as outlined in the methods section. The WAVE allows comparison of the DNA sample and a control that contains DNA with greater than 50% of the amplicons carrying the mutation and another control with 5% of the amplicons carrying the mutation, as well as a control without the mutation. A positive result is detected if the sample matches the peak pattern of the mutation controls.

Prior to running the patient samples a dilutional series of DNA samples were run by direct sequencing and utilizing the WAVE technology. The dilutional series included DNA samples of mutant alleles ranging from 2.5 to 100%. Direct sequencing without peptide nucleic acids (PNAs) mutation enrichment was able to detect mutant allele down to 20%. PNA mutant enrichment facilitates improved sensitivity of the sequencing, because the mutant enrichment PCR contains a PNA probe that blocks wild type KRAS amplification, but allows for mutant amplification if mutants are present. Direct sequencing with PNA mutation enrichment was able to detect mutant allele down to 2.5%; WAVE technology was able to detect mutant allele down to 2.5%. (see figure 1)

Eight samples from patient 1 were analyzed. DNA concentrations ranged from 30.5 – 84.8 ng/μL. In five of eight samples (B3, B4, B6, B7, B8 and B10) there is a clear peak pattern association between the sample and the mutation controls. One of eight (B10) demonstrates a poorly resolved, low amplitude peaks with a similar peak pattern as the mutation controls. Two of eight (B5 and B9) do not demonstrate any similarity in peak pattern and therefore are considered wild type. (See figure 2) All four samples from patient 2 tested positive for Exon 12/13 mutations. DNA concentrations for these samples ranged from 38.1 to 105.4 ng/μL. (See figure 3)

Samples from patient 3 had DNA concentrations ranging from 9 – 103ng/μL. Of the seven samples, four displayed poorly resolved, low amplitude peaks with retention times similar to KRAS fragments detected in controls known to carry KRAS exon 12/13 mutations (A1, A3, A5, B1). One sample demonstrated peaks with retention times and peak heights identical to positive controls (B3). Similar peaks were absent from the chromatographs of the remaining two samples (A4, A6). (See figure 4)

In order to insure that DNA concentration was not affecting the WAVE analysis, correlation with DNA concentration and the peak patterns were compared. In table 1, all DNA concentrations are listed and when compared to the tracings. Even at concentrations as low as 9ng/ul, retention times of mutant KRAS fragments as demonstrated by the WAVE results (peak patterns following out to 15 minutes which was the same as all three controls) were unaffected by DNA concentration.

Confirmation with sequencing

In order to confirm the findings on the WAVE, the remaining DNA samples were then analyzed utilizing standard molecular gene sequencing protocol, and if available the initial DNA was reevaluated. By standard sequencing (without PNA reaction) confident detection of the mutant allele can be performed if it's present in at least 20% of the DNA that we amplify. In order to improve the sensitivity of the sequencing, a mutant enrichment PCR is run along with a standard PCR. The mutant enrichment PCR contains a PNA probe that blocks wild type KRAS amplification, but allows for mutant amplification if mutants are present. When sequencing the PNA mutant enrichment PCR products, a KRAS mutant patient will appear homozygous mutant because we blocked wild type amplification. Wild type DNA generally does not amplify at a high enough efficiency to sequence from the mutant enrichment reaction. A sample is mutant if the amplification is relatively similar with or without the PNA meaning that there was not much inhibition by the PNA. The samples, that demonstrated wild type status by WAVE technology, were analyzed by standard direct sequencing PCR. The cases that were evaluated by direct sequencing demonstrated extremely low levels of mutant signal. The direct sequencing results without mutant enrichment appeared completely wild type, indicating a mutant frequency significantly below 20%. The PNA enrichment reaction appeared heterozygous, which is rare since typically we see a completely homozygous mutant peaks in mutant PNA reactions.

In sample one, four of the block samples were reevaluated by direct sequencing PCR. Of the four blocks, B4 and B6 were found to carry a KRAS mutation by WAVE technology, however when evaluated by direct sequencing only block B4 demonstrated the mutation. Block A5 and B9 were also evaluated by direct sequencing PCR due to lack of mutation detection by the WAVE, when checked by direct sequencing no mutation was also noted. The DNA utilized at time of clinical diagnosis taken from block B9 was available and reevaluated. On repeat direct sequencing PCR it was consistent with exon 12/13 mutation (figure 5)

In sample two, mutations were noted in all blocks therefore confirmation by direct sequencing was not repeated. In sample three, blocks A4, A5 and B1 did not demonstrate a mutation by WAVE analysis. When reevaluated by direct sequencing, no mutation was detected in any of the block samples.

Discussion

Cancer genome sequencing efforts are leading to the identification of an abundance of mutated genes in many cancer types. In CRC, the KRAS protein is considered a therapeutic target. The clinical efficacy of EGFR inhibitors in patients with KRAS wild type CRC

tumors is limited with response rates are as low as 15–20%. [10]The molecular bases of this clinical resistance to cetuximab in CRC remain poorly understood.

Polymerase chain reaction (PCR) has become the cornerstone of molecular diagnostic tools. PCR assays are highly sensitive and easily automated, thus well suited for large-scale high-throughput diagnostic testing. The sensitivity of a PCR assay depends upon three factors: the physicochemical conditions of the reaction, the concentration and nature of the DNA target and the selected PCR primers and probes. In KRAS testing standard PCR assays are not sufficient, because of the need to discriminate between different mutant alleles and wild type. By a variety of PCR methods, mutation detection limits of 5% allele abundance have been achieved. [11, 12] Levels as low as 1–2% have also been demonstrated by limiting the target size, performing reference replicates, using indexing tags and increasing sequence, but use of these methods in mainstream laboratory use is limited by expense and time. [13]When evaluating colon tissue the main challenges are tissue heterogeneity resulting from variability in the amount of tumor versus non-tumor tissue and differences in the detection limits for different mutations. [1]Variation in the location of the mutation in the tumor tissue in regards to KRAS mutational status has been demonstrated when paired primary tumors and metastases have been genotyped, at a rate of 7 to 11.4%. [14–16]

Is the WAVE more sensitive than pcr?

Prior to running the patients' samples a detection rate of 2.5% utilizing the WAVE was demonstrated. When the same dilutional series of DNA products was run by direct sequencing without mutant enrichment, the mutation detection rate was 20%. Others have demonstrated increased sensitivity of WAVE technology. Janne and colleagues evaluated 178 non-small cell lung cancer specimens for mutations in exons 18 to 21 of EGFR mutations. When compared to sequencing, the Transgenomic WAVE nucleic acid fragment analysis system demonstrated a sensitivity and specificity of 100% and 87% respectively; and detected mutation levels of 4%, sensitivity not previously obtained by direct sequencing. [9] As this data demonstrates, the WAVE technology permits a higher level of sensitivity and a lower level of ambiguity than direct sequencing of PCR products. In our analysis this was demonstrated in two separate findings. In sample one, four of the block samples were reevaluated by direct sequencing PCR. Of the four blocks, B4 and B6 were KRAS mutation positive by WAVE analysis, however when re-evaluated by direct sequencing only block B4 demonstrated the KRAS mutation.

Is KRAS presence heterogeneous in the tumor tissue?

In the advent of personalized medicine, etiologies for tumor resistance have raised the question of the role of tumor heterogeneity. Gerlinger and colleagues analyzed the intra-tumor heterogeneity of renal cell carcinoma. In their analysis, they performed whole exome multiregional spatial sequencing on 4 patients with metastatic renal cancer. For each patient, multiple regions in the primary tumor and metastatic tissue were analyzed. Evaluation included somatic mutational analysis, ploidy profiling and chromosomal aberration. After multi-region sequencing of four patients, 33–35% of all mutations were detected and present in all regions and all tissues. The only mutation maintained in all was in von Hippel Lindau gene. These findings eloquently demonstrate intra-tumor genomic heterogeneity and the

underestimation by recent genomic analyses of single tumor-biopsy specimens to demonstrate the mutational burden of heterogeneous tumors.

Much like Gerlinger and colleagues, the data generated in this study, supports the presence of intra-tumor heterogeneity within colorectal tumor tissue, a finding with a number of important clinical ramifications. In current practice, KRAS mutation analysis is usually performed on a single block of tissue. As demonstrated in samples 1 and 3 (2 of 8 and 2 of 7, respectively) there were areas of the tumor in which KRAS mutation were not detected, a heterogeneity which was missed by examining one block. This was also clearly illustrated by sample 1, A9 in which direct sequencing PCR demonstrated a clear mutation however on analysis by WAVE of another area of the same block no mutation was seen. Also supporting the heterogeneity of KRAS in regions of the tumor, is the WAVE data demonstrating variability of amplitude in peak patterns that suggesting a quantitative heterogeneity of the mutation presence within the tumor tissue. And finally, when utilizing the WAVE to analyze DNA extracted from multiple sites of a single tissue block, results demonstrated areas both positive and negative for mutated KRAS. This region variability in the presence of mutated KRAS presents a compelling argument to analyze multiple blocks or multiple areas within a tissue block, particularly if the results will dictate subsequent treatment.

Currently in breast and gastric cancer, FISH and IHC are utilized to detect HER2 presence. In the application of IHC, a grading system has been developed to provide clinically useful guidelines in regards to use of HER2 receptor inhibitors. FISH has been utilized as a confirmatory test. At this time the only protein that is guiding treatment in CRC is KRAS and the diagnostic modality that is directing treatment is PCR direct sequencing of DNA amplified from one area of the tumor. IHC and FISH modalities provide a histologic landscape of a protein's presence within a tumor. When utilizing PCR or the WAVE, data is limited to the presence of the mutation and not its distribution within the tumor. However, the areas of the tissue block that are utilized to obtain DNA product can provide a geographic landscape.

This data demonstrates that the Transgenomic WAVE nucleic acid fragment analysis system provides a robust, straightforward and highly sensitive mutation assay. In the targeted therapy paradigm of colorectal cancer, the heterogeneity of KRAS is clinically significant. In the BOND-2 phase II trial comparing cetuximab and bevacizumab with and without irinotecan demonstrated improvements in response rates. [17] However, subsequent studies the PACCE and CAIRO2 trials, demonstrated inferior progression free survival at a level that was statistically significant. [18, 19] On subset analysis of CAIRO-2, even the patients with wild-type KRAS tumors did not benefit from the addition of cetuximab. On review of these trials, the protocol for processing the tissue for DNA extraction consisted of real-time PCR based assay to assess presence of codon 12 and 13, and direct sequencing of exon 2 was performed to confirm results. [19] Current practice is to perform direct sequencing of exon 2 which is what was done in this study. This data suggests that interpretation of aberrant outcomes may be clinically informative of the heterogeneous expression of mutated KRAS in colorectal tissue. This data reinforces the concept that the heterogeneity of KRAS in colorectal cancer tissue likely dictates drug resistance.

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Figure 1B

13-00012KRAS codon 12 mutation detection LOD
(SW480 in Placental DNA background)

PNA Mutant Enrichment Real Time PCR Screening Assay

Target	CP A	CP B	Delta CP	7-Delta CP	Result
SW480	21.92	20.68	1.24	5.76	Positive
40%SW480	23.15	20.86	2.29	4.71	Positive
20%SW480	24.28	20.96	3.32	3.68	Positive
10%SW480	25.52	21.08	4.44	2.56	Positive
5%SW480	26.7	21.12	5.58	1.42	Positive
2.5%SW480	27.74	21.13	6.61	0.39	Positive
1.25%SW480	28.63	21.13	7.5	-0.5	Negative
Placenta	33.03	21.13	11.9	-4.9	Negative

Figure 1C

Figure 1. Dilution series utilizing the WAVE

A) Represented here is the WAVE derived peak pattern of DNA product of 3 mutation concentrations: Green (1734) is the control (>50% mutation), blue is 5% mutation, red is 2.5% mutation and Black (1975) is DNA wild type. This series of dilutions was performed to determine the sensitivity of the WAVE when compared to standard direct sequencing without PNA mutant enrichment which is only sensitive to a concentration of 20% mutation.

B) KRAS positive and negative samples are determined by subtracting the crossing point value of the “no PNA reaction” from the “PNA reaction” (delta CP). If the difference in reaction efficiency between the no PNA and PNA reactions is less than or equal to 7 crossing points (Delta CP ≤ 7), then the sample is considered positive for a KRAS codon 12 or 13 mutation. Both reactions are then sequenced to confirm and determine the exact mutation. Here, the 2.5% SW480 sample appears positive, but the 1.25% SW480 is negative. Samples in this range are not consistently positive, so we do not claim such a low sensitivity.

C) These are the traces obtained from sequencing the PCR product obtained from the above PNA screening PCR. Boxed off in red, are the traces where the correct mutation is still confidently observed- 1.5% for the “with PNA” reaction and 20% for the “without PNA” reaction.

Figure 2.

Wave results from Sample 1. The WAVE allows comparison of the DNA sample and a control that contains the mutation at >50% and at 5%, as well as a control without the mutation (Wild Type). A positive result is detected if the sample matches the peak pattern of the mutation controls. B5 and B9 do not demonstrate a similarity when compared to 5% control (black). All other samples share a similar tracing pattern when compared to the 5% control (black).

Figure 3.

Sample 2. The WAVE allows comparison of the DNA sample and a control that contains the mutation at >50% and at 5%, as well as a control without the mutation (Wild Type). A positive result is detected if the sample matches the peak pattern of the mutation controls. These samples demonstrate similar appearance in all sample tracings when compared to the control, therefore consistent with KRAS exon 12/13 mutation.

Figure 4.

Sample 3. The WAVE allows comparison of the DNA sample and a control that contains the mutation at >50% and at 5%, as well as a control without the mutation (Wild Type). A positive result is detected if the sample matches the peak pattern of the mutation controls.

A) results consistent with KRAS mutation presence are demonstrated in A1, A3, A5, B1 and B3.

B) lack of KRAS mutation noted in A4 and A6

Figure 5.

These images represent direct sequencing PCR results when compared to WAVE results for the same patient and the same block. Positive mutation results are seen when the mutation is present without the PNA mutant enrichment. These results confirm mutation absence and heterogeneity within the same tumor tissue block utilizing standard direct sequencing methods in patient one.

Table 1

DNA PCR product concentrations for samples 1, 2 and 3.

CRC 19	
Sample 1	Concentration (ng/ul)
3	63.3
4	77.0
5	30.5
6	84.8
7	61.2
8	36.1
9	55.8
10	50.5

CRC 21	
Sample 2	Concentration (ng/ul)
A2	38.1
A4	105.4
A5	53.9
A13	59.3

CRC 20	
Sample 3	Concentration (ng/ul)
A1	20.2
A3	103.0
A4	9.0
A5	31.6
A6	15.2
B1	55.8
B3	81.7