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Performance characteristics of next-generation sequencing in clinical mutation detection of colorectal cancers

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Activating mutations in downstream genes of the epidermal growth factor receptor (EGFR) pathway may cause anti-EGFR resistance in patients with colorectal cancers. We present performance characteristics of a next-generation sequencing assay designed to detect such mutations. In this retrospective quality assessment study, we analyzed mutation detected in the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes by a clinically validated next-generation sequencing assay in 310 colorectal cancer specimens. Tumor cellularity and mutant allele frequency were analyzed to identify tumor heterogeneity and mutant allele-specific imbalance. Next-generation sequencing showed precise measurement of mutant allele frequencies and detected 23% of mutations with 2–20% mutant allele frequencies. Of the *KRAS* mutations detected, 17% were outside of codons 12 and 13. Among *PIK3CA* mutations, 48% were outside of codons 542, 545, and 1047. The percentage of tumors with predicted resistance to anti-EGFR therapy increased from 40% when testing for only mutations in *KRAS* exon 2 to 47% when testing for *KRAS* exons 2–4, 48% when testing for *KRAS* and *NRAS* exons 2–4, 58% when including *BRAF* codon 600 mutations, and 59% when adding *PIK3CA* exon 20 mutations. Right-sided colorectal cancers carried a higher risk of predicted anti-EGFR resistance. A concomitant *KRAS* mutation was detected in 51% of *PIK3CA*, 23% of *NRAS*, and 33% of kinase-impaired *BRAF*-mutated tumors. Lower than expected mutant allele frequency indicated tumor heterogeneity, while higher than expected mutant allele frequency indicated mutant allele-specific imbalance. Two paired neuroendocrine carcinomas and adjacent adenomas showed identical *KRAS* mutations, but only *PIK3CA* mutations in neuroendocrine carcinomas. Next-generation sequencing is a robust tool for mutation detection in clinical laboratories. It demonstrates high analytic sensitivity and broad reportable range, and it provides simultaneous detection of concomitant mutations and a quantitative measurement of mutant allele frequencies to predict tumor heterogeneity and mutant allele-specific imbalance.
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Epidermal growth factor receptor (EGFR) regulates cell differentiation, proliferation, and survival via the mitogen-activated protein kinase (MAPK or RAS/RAF/MEK/ERK) pathway and the phosphatidylinositol 3-kinase (PI3K/AKT/mTOR) pathways.[1] Cetuximab and panitumumab are anti-EGFR monoclonal antibodies approved by the Food and Drug Administration (FDA) for treatment of patients with *KRAS*-wild-type metastatic colorectal cancers, but the response rate is only ~10% in unselected patient populations.[2,3] This poor response to anti-EGFR therapy can be explained, in part, by activating mutations in genes of the downstream signaling pathways, such as *KRAS* or *NRAS*.[4–6]

In 2009, a provisional clinical opinion from the American Society of Clinical Oncology recommended testing for *KRAS* gene mutations in patients with metastatic colorectal cancers to predict response to anti-EGFR therapy.[7] In addition to common *KRAS* mutations at codons 12 and 13, recent studies have shown that less common mutations at codons 59, 61, 117, and 146 and mutations of the *NRAS* gene also predict anti-EGFR resistance.[5–9] *BRAF* mutations may also predict resistance to anti-

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EGFR therapy, although more prospective studies are needed to confirm these findings.[5,10] In colorectal cancers, mutations in the *PIK3CA* gene are usually accompanied by a *KRAS* mutation.[5,11] The benefit of testing *PIK3CA* mutations, particularly exon 20 mutations, to guide anti-EGFR therapy therefore requires further studies in a large cohort of colorectal cancer patients with wild-type *KRAS* and *NRAS* genes. The revised guideline for anti-EFGR therapy in 2014 from National Cancer Comprehensive Network recommends testing at least *KRAS* mutations at codons 12 and 13 as well as, whenever possible, additional *KRAS* mutations outside of exon 2, as well as *NRAS* mutations. It recommends considering *BRAF* testing if both *KRAS* and *NRAS* are nonmutated. The Association of Clinical Pathologists Molecular Pathology and Diagnostics Group in the United Kingdom also recommends that RAS analysis should include at least codons 12, 13, 59, 61, 117, and 146 of the *KRAS* gene and codons 12, 13, 59, and 61 of the *NRAS* gene.[12]

Massively parallel sequencing or next-generation sequencing technology has led to a revolution in genome discovery. The convergence of recent advances in molecular technology and rapid expansion of targeted therapeutics have changed approaches in clinical molecular diagnostics laboratories from the traditional 'one test-one drug' paradigm to the multiplexed genotyping platform that are used to simultaneously test a panel of genes for a specific tumor type.[13,14] Next-generation sequencing will soon become the most cost-effective multiplexed sequencing platform in the setting of clinical care as more and more biomarkers become mandatory clinical tests.[13] Next-generation sequencing assays have also been retrospectively used to evaluate response to anti-EGFR therapy in colorectal cancer patients.[8,15] We have previously validated a next-generation sequencing platform using the AmpliSeq Cancer Hotspot Panel and Personal Genome Machine in a Clinical Laboratory Improvement Amendments-certified laboratory.[16] In this retrospective analysis for quality assessment, we survey our experience with clinical mutation detection in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes in 310 colorectal cancer specimens using next-generation sequencing.

Materials and methods

A total of 314 formalin-fixed paraffin-embedded specimens with a diagnosis of colorectal cancer were submitted to the Molecular Diagnostics Laboratory at The Johns Hopkins Hospital between April 2013 and November 2014. The test was canceled in four specimens; two with scant tumor cells, one referral case with no tumor cells on the accompanying hematoxylin & eosin (H&E) slide, and one with the assay being successfully conducted in the primary tumor. The remaining 310 specimens were submitted from 305 tumors of 301 colorectal cancer patients (Supplementary Table S1). There were 258 resection specimens, 49 biopsy specimens, and three fine-needle aspiration specimens (Supplementary Table S2). Tissue blocks with adequate tumor cellularity were selected by the pathologists who made the diagnosis. Almost all were diagnosed as invasive adenocarcinoma, with the exceptions being one with neuroendocrine carcinoma (case 54) and one with mixed adenocarcinoma and neuroendocrine carcinoma (case 282). One H&E slide followed by 5–10 unstained slides and one additional H&E slide were prepared with PCR precautions. The H&E slide was examined and marked by pathologists for subsequent macro-dissection of formalin-fixed paraffin-embedded neoplastic tissues from 3–10 unstained slides of 5 or 10-μm-thick sections. DNA was isolated from the area(s) designated by pathologists using the Pinpoint DNA Isolation System (Zymo Research, Irvine, CA, USA), followed by further purification via the QIAamp Mini Kit (Qiagen, Valencia, CA, USA).[17] The estimated percentage of tumor nuclei was separated into 5 quintiles (1–20%, 21–40%, 41–60%, 61–80%, and 81–100%) by two pathologists (GZ and MTL). In the presence of discrepancy, the mean value was applied.

Next-generation sequencing was conducted using AmpliSeq Cancer Hotspot Panel (v2) for targeted multi-gene amplification, as described previously.[16] Briefly, we used the Ion AmpliSeq Library Kit 2.0 for library preparation, Ion OneTouch 200 Template Kit v2 DL and Ion OneTouch Instrument for emulsion PCR and template preparation, and the Ion Personal Genome Machine 200 Sequencing Kit with the Ion 318 Chip and Personal Genome Machine as the sequencing platform (Life Technologies, Carlsbad, CA, USA). The DNA input was up to 30 ng, as measured by Qubit 20 Fluorometer (Life Technologies). Up to eight specimens were barcoded using Ion Xpress Barcode Adapters (Life Technologies) for each Ion 318 chip. One to three controls (a non-template control, a normal peripheral blood control from a male, and/or positive control specimens) were included in each chip. Positive controls were mixed DNA specimens from several cell lines with known mutations (Supplementary Table S3).

Mutations were identified and annotated through both Torrent Variant Caller (Life Technologies) and direct visual inspection of the binary sequence alignment/map file using the Broad Institute's Integrative Genomics Viewer. All specimens were analyzed for *BRAF*, *KRAS*, *NRAS*, and *PIK3CA* genes. During our validation of this next-generation sequencing assay, a cutoff of background noise at 2% was chosen for single-nucleotide variations according to a study of 16 non-neoplastic formalin-fixed paraffin-embedded tissues.[16] We also developed a statistical model to determine the read depth needed for a given percent tumor cellularity and number of functional genomes. With sufficient DNA input, the limit of detection is dictated by the depth of coverage (or number of sequencing reads). Approximately 150

and 500 reads is needed to detect a heterozygous mutation at a 99% confidence in a specimen with 20 and 10% tumor cellularity, respectively. The reportable ranges and reference ranges are summarized in Supplementary Table S4.

Pyrosequencing and SNP array analysis was performed as previously described.[18] Correlation between % KRAS mutant *versus* % *PIK3CA* mutant was examined by Spearman's rank correlation coefficient (denoted as *r*) using the GraphPad Prism software (GraphPad Software, ver5, La Jolla, CA, USA).

Results

Positive and Negative Controls

No mutation was detected in 88 runs of the negative control specimen and all mutations in the positive control specimens were detected. The observed mutant allele frequencies were highly consistent throughout the test period, demonstrating that next-generation sequencing is a precise quantification assay for mutant allele frequency (Figure 1 and Supplementary Table S3).

KRAS, NRAS, BRAF, and PIK3CA Mutations in Colorectal Cancers

Two of 310 specimens failed (0.6%), including one of two specimens from different blocks of a tumor. Five paired specimens from the same tumor showed identical mutation status. Next-generation sequencing data, therefore, were available for analysis in 304 tumors (Supplementary Table S1). Mutations were not detected in any of the *KRAS*, *NRAS, BRAF,* and *PIK3CA* genes (quadruple mutation-negative) in 98 (32%) tumors. One mutation was detected in 167 tumors, two mutations in 36 tumors, and three mutations in three tumors. Because our analysis pipeline included both Torrent Variant Caller and direct visual inspection of all reported amplicons using Integrative Genomics Viewer, three mutations (8.8% *PIK3CA* p.V344M [c.1030G>A], 1.95% *KRAS* p.Q61H [c.183A>C], and 40% *BRAF* p.Y472C [c.1415A>G]) were detected by Integrative Genomics Viewer inspection but missed by Torrent Variant Caller.

Next-generation sequencing was retrospectively performed in two specimens that had been canceled because of insufficient tumor cells on the H&E slides. DNA was extracted from 7 to 10 unstained slides and next-generation sequencing assays conducted with 0.8 and 1.8 ng DNA, respectively. One specimen showed 19% *KRAS* p.A146T (c.436G>A), 34% *PIK3CA* p.V344M (c.1030G>A), and 43% *PIK3CA* p.H1047R (c.3140A>G) mutations and the other a 37% *KRAS* p.G12C (c.34G>T) mutation. The overall assay success rate was 310 out of 312 (99.4%).

A total of 20 unique *KRAS* mutations were detected in 146 out of 304 tumors (48%), including one tumor with both p.G12A (c.35G>C) and p.G13D (c.38G>A) mutations. The mutations were located in exon 2 in 123 tumors (41%), exon 3 in 10 tumors (3.3%) and exon 4 in 13 tumors (4.3%). p.G12D (c.35>G>A), p.G12V (c.35G>T), and p.G13D (c.38G>A) were the most common *KRAS* mutations in colorectal cancers (Supplementary Figure S1). Mutations located outside codon 12 or 13 were observed in 25 tumors (17% of *KRAS* positive tumors). Six unique *NRAS* mutations were detected in 13 tumors (4.3%), 5 at codon 12 or 13 and 8 at codon 61. Five unique *BRAF* mutations were detected in 36 tumors (12%), including 28 p.V600E (c.1799T>A) mutations with predicted highly elevated BRAF kinase activity, 2 p.N581S mutations with unknown kinase activity, and six mutations (4 p.D594G [c.1781A>G], one p.D594N [c.1780G>A] and one p.Y472C [c.1415A>G]) with predicted reduced or silent kinase activity.[19,20]

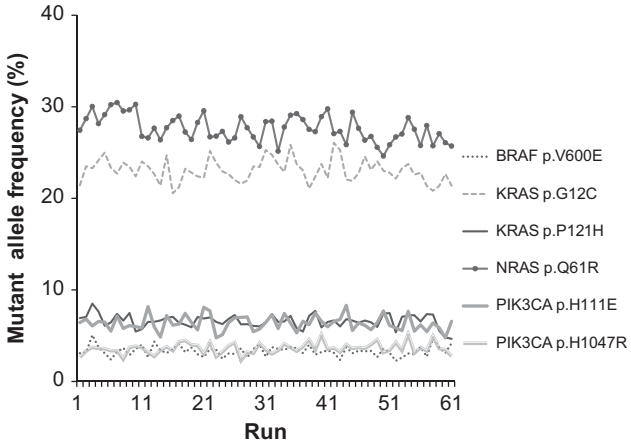

Figure 1 Precision of mutant allele quantification by next-generation sequencing. Mutant allele percentage detected in a positive control specimen was highly consistent across 38 runs.

Twenty-three unique *PIK3CA* mutations were detected in 47 tumors (16%), including five tumors with double *PIK3CA* mutations and 17 tumors with mutations detected only in the *PIK3CA* gene. The *PIK3CA* exon 20 mutation, most associated with resistance to EGFR therapy, without concomitant *KRAS*, *NRAS*, and *BRAF* mutations was observed in only five tumors (1.6%). Most mutations were located within exons 9 (42%), 20 (23%), and 1 (17%) (Supplementary Figure S2). All but p.E110del (c.328_330del) and p.E453del (c.1359_1361del) mutations were single-base substitution, including a p.E81A (c.242A>C) mutation not reported in the COSMIC database (last access on 14 May 2015). The three most common codons (E542, E545, and H1047) account for only 27 of 52 (52%) *PIK3CA* mutations.

Prediction of Resistance to Anti-EGFR Therapy in the Right- and Left-Sided Colorectal Cancers

Cancers proximal or distal to the splenic flexure were classified as right-sided or left-sided. The

Table 1 *KRAS*, *NRAS*, *BARF,* and *PIK3CA* mutations in right-sided and left-sided colorectal cancers

Location	*Quadruple negative*[a]	*KRAS*	*NRAS*	*BRAF*	*MAPK*[b]	*PIK3CA*	*Only PIK3CA*
Right ($n=116$)	16 (14%)	64 (55%)	9 (8%)	26 (22%)	94 (81%)	20 (17%)	6 (5%)
Left ($n=176$)	78 (44%)	75 (43%)	4 (2%)	9 (5%)	87 (49%)	25 (14%)	11 (6%)
	$P<0.001$	$P=0.04$	$P=0.04$	$P<0.001$	$P<0.001$	$P=0.48$	*$P=0.70$*

[a]No mutation detected in the *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes. [b]Mutations in the *KRAS*, *NRAS,* and/or *BRAF* genes, including three tumors with concomitant *KRAS* and *NRAS* mutations and two tumors with concomitant *KRAS* and *BRAF* mutations in the right-sided colorectal cancers, and one tumor with concomitant *KRAS* and *BRAF* mutations in the left-sided colorectal cancers.

primary tumors were located within the right colon in 116 (38%) tumors and the left colorectum in 176 (58%) tumors. The exact location of the primary tumor was not known in nine tumors and the exact location of the primary tumors within the transverse colon was not known in three tumors. Quadruple mutation-negative tumors were observed in only 16 right-sided colorectal cancers and in 78 left-sided colorectal cancers (14 *vs* 44%, $P<0.001$) (Table 1). Right-sided colorectal cancers showed a significantly higher incidence of mutations in *BRAF*, *KRAS*, *NRAS* genes within the MAPK pathway (Table 1). Cecal cancer exhibited the highest frequency of *RAS* mutations (63 *vs* 51% of non-cecum right-sided colorectal cancers, 41% of non-rectum left-sided colorectal cancers, and 47% of rectal cancers) and the lowest frequency of quadruple mutation-negative tumors (12 *vs* 19% of non-cecum right-sided colorectal cancers, 48% of non-rectum left-sided colorectal cancers, and 42% of rectal cancers).

Resistance to anti-EGFR therapy has been associated with mutations at codons 12, 13, 59, 61, 117, and 146 of the *KRAS* and/or *NRAS* gene, and to a lesser extent, p.V600E mutations of the *BRAF* gene and exon 20 mutations of the *PIK3CA* gene.[4–6] The predicted population with lack of response increased from 40% when testing for only mutations in *KRAS* exon 2 to 47% when testing for *KRAS* exons 2–4, 48% when testing for *KRAS* and *NRAS* exons 2–4, 58% when including *BRAF* codon 600 mutations, and 59% when adding *PIK3CA* exon 20 mutations (Figure 2). The right-sided colorectal cancers carry a higher risk of containing resistance mutations to anti-EGFR therapy (79 *vs* 49%, $P<0.001$). The difference between right-sided and left-sided colorectal cancers was not significant when only *KRAS* codons 12 and 13 mutations were examined (43 *vs* 36%, $P=0.25$).

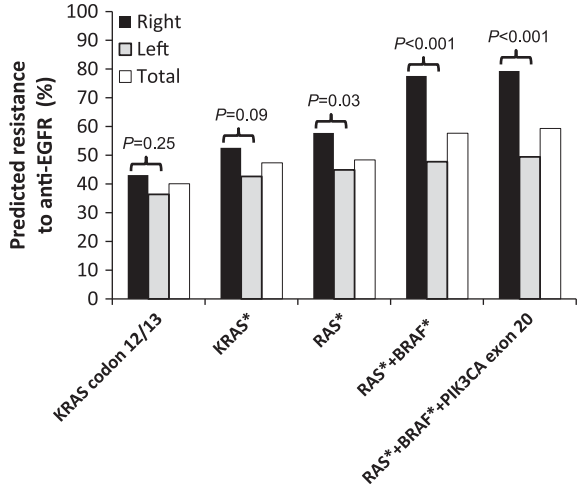

Figure 2 Prediction of resistance to anti-epidermal growth factor receptor therapy (*y* axis) according to reportable ranges of assays (*x* axis). *KRAS**: codons 12, 13, 59, 61, 117, and 146 of the *KRAS* gene; RAS*: codons 12, 13, 59, 61, 117, and 146 of the *KRAS* and *NRAS* genes; BRAF*: codon 600 of the *BRAF* gene.

Mutant Allele Frequency as Quality Assessment Tool to Identify Tumor Heterogeneity

A total of 251 mutations were detected in the 308 specimens with next-generation sequencing data. The mutant allele frequency was 2–5% in six out of 251 mutations (2.4%), 5–10% in 14 mutations (5.6%), and 10–20% in 38 mutations (15%). Among the 20 specimens with 10% or fewer mutant alleles, the mutant allele percentage was consistent with a 1–20% or 11–30% estimated tumor cellularity in 12 specimens (Table 2). These included one lymph node specimen with an infiltrative metastasis (case 1) (Supplementary Figure S3a), three small biopsy specimens (cases 67, 174, and 227) and one resected specimen (case 76) with tumor cells surrounded by prominent desmoplastic reaction and/or inflammatory cell infiltration (Supplementary Figure S3b), one resected mucinous adenocarcinoma (case 270) (Supplementary Figure S3c), and five resected specimens of rectal cancer (cases 75, 98, 158, 217, and 287) and one resected specimen of liver metastasis (case 213) with prior chemo/radiotherapy and/or embolization therapy (Supplementary Figure S3d).

Mutations with an observed allele frequency at least 10 percentage points lower than that expected from the estimated tumor cellularity were observed in eight specimens, suggesting that these mutations are present in a subpopulation of the neoplasm. DNA samples isolated from several subpopulations within tumors demonstrated intra-tumor heterogeneity for the *KRAS* mutation in case 33 (Supplementary Figure S4) and the presence of *NRAS* and *PIK3CA* mutations in different mesentery tumor nodules in case 127 (Supplementary Figure S5). In case 282 with a cecal mixed adenocarcinoma and neuroendocrine carcinoma, a 53% *KRAS* p.A146T (c.436G>A) mutation

Table 2 Specimens with 10% or lower mutant allele frequencies

Case	*Specimens*	*Tumor %*	*mut 1*	*mut 1 (%)*	*mut 2*	*mut 2 (%)*
1	LN/resection	1–20	G12D/*KRAS*	3.5		
2	Rectum/resection[a]	31–50[b]	V344M/*PIK3CA*	8.8[b]		
33	Colon/resection	31–50[b]	Q61H/*KRAS*	1.95[b]		
67	Colon/biopsy	1–20	E545K/*PIK3CA*	7.7	G12D/*KRAS*	17
75	Rectum/resection[a]	1–20	D594G/*BRAF*	7.2		
76	Colon/resection	1–20	Q546H/*PIK3CA*	4.1	G12D/*KRAS*	14
91	Colon/resection	41–60[b]	E81K/*PIK3CA*	8.9[b]	E545K/*PIK3CA*	13[b]
98	Rectum/resection[a]	1–20	G12A/*KRAS*	9.9		
127	Colon/resection	51–70[b]	Q61R/*NRAS*	4.0[b]	H1047R/*PIK3CA*	14[b]
144	Colon/biopsy	41–60[b]	G15S/*KRAS*	8.4[b]	V600E/*BRAF*	29
158	Rectum/resection[a]	1–20	A59G/*KRAS*	5.9		
174	Colon/biopsy	1–20	E542K/*PIK3CA*	6.9		
202	Colon/resection	61–80[b]	G12V/*KRAS*	5.2[b]	G13C/*NRAS*	36
213	Liver/resection[a]	11–30	G12D/*KRAS*	6.4		
217	Rectum/resection[a]	1–20	N345K/*PIK3CA*	6.9		
227	Liver biopsy	11–30	V600E/*BRAF*	7.5		
270	Colon/section	11–30	E110del/*PIK3CA*	9.4	G13D/*KRAS*	17
282	Colon/resection	61–80[b]	E545K/*PIK3CA*	8.9[b]	A146T/*KRAS*	53
287	Rectum/resection[a]	1–20	A146T/*KRAS*	3.4		
302	Rectum/resection[a]	31–50[b]	E545K/*PIK3CA*	4.6[b]		

Abbreviations: mut, mutation; mut 1%, allele frequency of mutation 1; mut 2%, allele frequency of mutation 2; G12A, c.35G>C; G12D, c.35>G>A; G12V, c.35G>T; G13C, c.37G>T; G13D, c.38G>A; G15S, c.43G>A; A59G, c.176C>G; Q61H, c.183A>C; Q61R, c.182A>G; E81K, c.241G>A; E110del, c.328_330del; A146T, c.436G>A; V344M, c.1030G>A; N345K, c.1035T>A; E542K, c.1624G>A; E545K, c.1633G>A; Q546H, c.1638G>T; D594G, c.1781A>G; V600E, c.1742A>G; H1047R, c.3140A>G.
[a]Resection with prior adjuvant therapy.
[b]Mutations with an observed mutant allele frequency at least 10% less than that expected from the estimated tumor cellularity. The expected mutant allele percentage is half of the estimated tumor percentage, assuming heterozygosity with no mutant allele-specific imbalance.

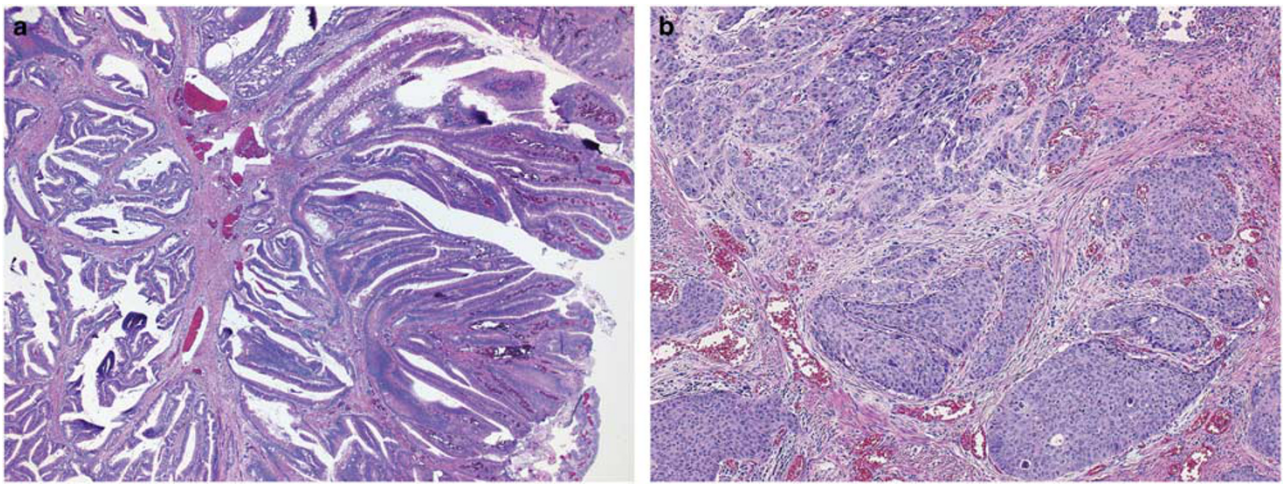

Figure 3 Invasive neuroendocrine carcinoma (**b**) and adjacent adenoma (**a**) in case 282. Original magnifications of the hematoxylin and eosin stained slides are ×10 for (**a**) and ×40 for (**b**).

and an 8.9% *PIK3CA* p.E545K (c.1633G>A) were detected in the originally isolated DNA specimen extracted from a designated area containing mainly the adjacent adenoma and a small component of the invasive neuroendocrine carcinoma (Figures 3 and 4). The *PIK3CA* mutation was detected only in the DNA re-isolated from the invasive neuroendocrine carcinoma, but not the adenoma (Figure 4).

Concomitant Mutations

Concomitant mutations within *KRAS, NRAS, BRAF,* and *PIK3CA* genes were observed in 39 out of 206 tumors (19%) with mutations (Table 3). These included one tumor with two different *KRAS* mutations, three tumors with a *KRAS* mutation and two *PIK3CA* mutations, two tumors with two *PIK3CA* mutations, and 33 tumors with two mutations of different genes. Tumors with a *PIK3CA* mutation showed a significantly higher incidence of concomitant mutations of different genes (64%) as compared with tumors with a *KRAS* (21%) or *BRAF* (19%) mutation ($P<0.001$) (Supplementary Figure S6). Concomitant *KRAS* mutation was seen in 24 out of 47 (51%) *PIK3CA*-mutated tumors, three out of 13 (23%) *NRAS*-mutated tumors, and two out of six

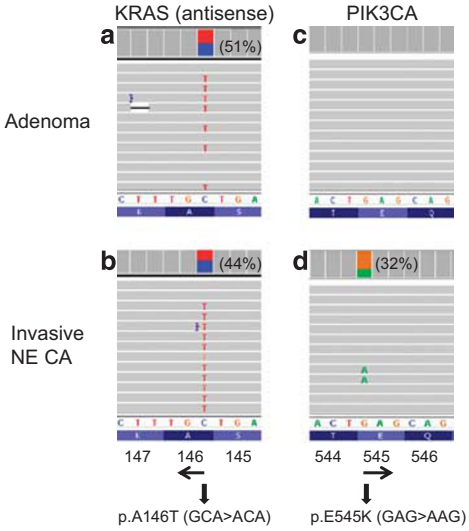

Figure 4 *PIK3CA* mutation in invasive neuroendocrine carcinoma but not adenoma. In case 282, tubular adenoma showed only a *KRAS* p.A146T (c.436G>A) mutation (**a** and **c**), while the invasive neuroendocrine carcinoma (NE CA) showed a *KRAS* p.A146T mutation and a *PIK3CA* p.E545K (c.1633G>A) mutation (**b** and **d**). Mutant allele frequency is shown in the parenthesis.

Table 3 Concomitant mutations of *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* genes

Genes	*Tumors*	*PIK3CA exon 9*	*PIK3CA exon 20*
KRAS+PIK3CA	24	17	4
BRAF+PIK3CA	4	1	2
NRAS+PIK3CA	2	0	1
PIK3CA+PIK3CA	2	1	0
KRAS+KRAS	1[a]		
KRAS+BRAF	3[b]		
KRAS+NRAS	3[c]		

[a]p.G12A and p.G13D. [b]*KRAS* p.G15S (c.43G>A) and *BRAF* p.V600E (c.1799T>A); *KRAS* p.A59E (c.176C>A) and *BRAF* p.D594G (c.1781A>G); and *KRAS* p.G12V (c.35G>T) and *BRAF* p.Y472C (c.1415A>G). [c]*KRAS* p.G12D (c.35>G>A) and *NRAS* p.Q61K (c.181C>A); *KRAS* p.A146T (c.436G>A) and *NRAS* p.Q61R (c.182A>G); and *KRAS* p.G12V (c.35G>T) and *NRAS* p.G13C (c.37G>T).

BRAF codon 594 or 472 mutated tumors. Concomitant mutations with *KRAS*, *BRAF*, or *NRAS* mutations were detected in 18 out of 22 (82%) tumors with a *PIK3CA* exon 9 mutation and in seven out of 12 (58%) tumors with a *PIK3CA* exon 20 mutation.

PIK3CA Mutation in the Invasive Neuroendocrine Carcinoma but Not the Adjacent Adenoma

In addition to case 282, DNA samples were also isolated from adjacent adenomas in four specimens with concomitant *KRAS* and *PIK3CA* mutations. The same mutations were detected in the adenoma and the originally examined invasive adenocarcinoma of cases 76, 106, and 108 (Table 4). Similarly to case 182, only a *KRAS* mutation was observed in the adenoma of case 54, even though both *KRAS* and *PIK3CA* mutations were detected in the originally examined invasive neuroendocrine carcinoma.

Table 4 Paired invasive carcinoma and adjacent adenoma specimens

	Mutant allele (%)			*KRAS*	*PIK3CA*
Case	*KRAS*	*PIK3CA*	*Expected*[a]	*SNP array*[b]	*SNP array*[b]
54ad	42	Not detected	31–40		
54iv[c]	55	37	31–40	Gain	Negative
76ad	50	33	31–40		
76iv[c]	14	4	1–10	Gain	Negative
106ad	53	35	31–40		
106iv[c]	48	23	31–40	Gain	Negative
108ad	57	28	31–40		
108iv[c]	52	24	26–35	Gain	Negative
282ad	51	Not detected	31–40		
282mix[c]	53	9	31–40		
282iv	44	32	21–30	Gain/LOH	Gain

Abbreviations: ad, adenoma; iv, invasive tumor; LOH, loss of heterozygosity; mixed, mixed adenoma and invasive tumors.
The invasive component was neuroendocrine carcinoma instead of adenocarcinoma in cases 54 and 282.
[a]The expected mutant allele percentage is half of the estimated tumor percentage for a heterozygous mutation.
[b]negative: no aneuploidy; gain/LOH (copy neutral LOH): duplication of one allele and loss of the other allele.
[c]DNA extracted for original mutation detection.

Prediction of Mutant Allele-Specific Imbalance Using Concomitant Mutant Allele Percentages

The extremely high concordance of mutant allele frequencies ($r=0.99$) in five tumors with two *PIK3CA* mutations and one tumor with two *KRAS* mutations suggests that the paired mutations of the same gene most likely occurred within the same tumor cells (Supplementary Figure S7). The *KRAS* and *PIK3CA* mutant allele frequencies in 24 tumors with concomitant mutations were also significantly correlated, but the degree of association was weaker ($r=0.56$) (Figure 5). The ratio of *PIK3CA*/KRAS mutant allele frequencies was within the range between 80 and 120% in only 10 tumors and was below 80% (17–67%) in 12 tumors. A *PIK3CA* mutant allele frequency lower than the KRAS mutant allele frequency suggests that (i) the *PIK3CA* mutation was present in a subpopulation of the tumor cells (as shown in case 282) or (ii) a higher incidence of amplification of the KRAS mutant allele or loss of the wild-type allele of the *KRAS* gene. This was confirmed by SNP array analysis from five invasive tumors showing no aneuplody of chromosome 3q containing the *PIK3CA* genes and gain of chromosome 12p12 containing the *KRAS* gene (Table 4 and Supplementary Figure S8).

Discussion

A variety of molecular assays have been developed for clinical detection of *KRAS* and *BRAF* mutations.

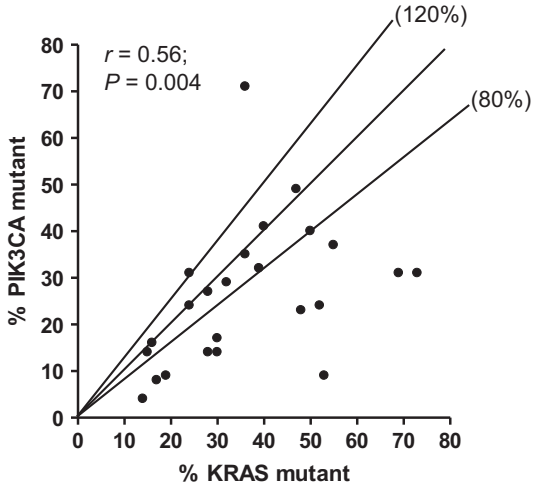

Figure 5 Correlation of mutant allele frequencies in tumors with concomitant *KRAS* and *PIK3CA* mutations. The lines labeled with 80 and 120% indicate the boundary of events with the *PIK3CA*/KRAS mutant allele ratio between 80 and 120%. Only one *PIK3CA* mutant allele was included for the three specimens with a *KRAS* mutation and two *PIK3CA* mutations. *r*: Spearman's rank correlation coefficient.

Specimens containing low tumor cellularity are not uncommon in the clinical diagnostic setting.[21,22] With an analytic sensitivity of 10–20% mutant allele frequency, the prior gold standard of Sanger sequencing would have missed 20 out of 251 (8%) mutations with $<10\%$ mutant allele frequency or 58 (23%) mutations with $<20\%$ mutant allele frequency in this series. This is particularly problematic for colorectal cancer specimens with prior neoadjuvant therapy.[22] The analytic sensitivity can be improved to ~5% mutant allele frequency by using pyrosequencing, high-resolution melting analysis or real-time PCR assays or even 1% with allele-specific PCR assays.[18,23–25] Cobas 4800 is a real-time PCR assay approved by the FDA of the United States for companion BRAF testing. The *Therascreen* KRAS RGQ PCR kit is an allele-specific PCR assay approved as a companion KRAS test.[23–25] However, cobos 4800 detects only the p.V600E mutation and non-p.V600E codon 600 mutations at a lower analytic sensitivity. Therascreen detects seven common *KRAS* mutations in 8 PCR reactions and would have missed 26 of 147 (18%) of *KRAS* mutations detected by next-generation sequencing in this study. In addition, these assays detect only one or a few hotspots in a single run and may not be suitable for some core biopsy or fine-needle aspiration specimens containing limited tissue.

In an era of continuous expansion of predictive markers for targeted therapeutics, multiplexed assays are becoming popular in clinical laboratories. Primer extension-based assays with a multiplex design, such as the Sequenom MassARRAY system, detect multiple hotspots in a single reaction while retaining an analytic sensitivity of 5% mutant alleles or less.[5,26] In this study, we demonstrated that next-generation sequencing provides not only a high analytic sensitivity (low limit of detection) but also a broad reportable range for simultaneous mutation detection of a panel of *KRAS*, *NRAS*, and potentially *BRAF* and *PIK3CA* genes relevant to anti-EGFR therapy of colorectal cancers in a clinical diagnostic setting. Twenty-five out of 147 (17%) *KRAS* mutations were located outside codons 12 and 13 and eight out of 36 (22%) *BRAF* mutations were located outside codon 600. Only 52% of *PIK3CA* mutations occurred at codons 542, 545, and 1047 with our next-generation sequencing assay. Application of assays with broader reportable ranges may shed light on the clinical significance of uncommon mutations. For example, response to anti-EGFR therapy has been observed in a patient with a p.D594G mutation, which encodes a BRAF protein with impaired kinase activity.[5,19] *BRAF* mutations with reduced or silent kinase activity do not directly activate the downstream MAPK pathway.[19,20] In the presence of oncogenic RAS proteins, however, kinase-silent *BRAF* forms a complex with *CRAF* and leads to hyperactivation of the CRAF/MEK/ERK cascade.[20] *BRAF* codon 594 mutations have been associated with a higher incidence of concomitant RAS mutations (4 in 34, 12%).[20] In our colorectal cancer population, concomitant *KRAS* mutations were observed in two out of six cases with *BRAF* mutations (one at codon 472 and five at codon 594) with impaired kinase activity.[19,27]

One study has suggested that *PIK3CA* exon 20 mutations may be predictive for anti-EGFR resistance,[5] but their significance is still debated, partly because of a low incidence of *PIK3CA*-only mutations.[11] In two recent meta-analyses of *KRAS* wild-type patients, only 12–14 patients with a *PIK3CA* exon 20 mutation could be enrolled for analysis.[28,29] In this study, only five (1.6%) tumors were positive for *PIK3CA* exon 20 mutation without concomitant *KRAS*, *NRAS*, and *BRAF* mutations. Like *PIK3CA* exon 20-only mutations, *NRAS*-only mutations were observed in only 4 (1.3%) tumors.

Common mutations of *KRAS*, *NRAS*, *BRAF,* and *PIK3CA* genes were examined using the Sequenom MassARRAY system in 773 patients treated with cetuximab.[5] The conditional inference tree analysis for response suggested that *KRAS* testing should be considered first, *BRAF* second, *NRAS* third, and *PIK3CA* exon 20 fourth. The response rate improved from 24% in an unselected population to 41% in the quadruple negative population, which accounted for 50% of the population. Using next-generation sequencing assays, which have a broader reportable range, only 32% of tumors (14% of right-side colorectal cancers and 44% of left-side colorectal cancers) were quadruple mutation-negative. The predicted anti-EGFR-resistant population increased from 40% when testing only *KRAS* codons 12 and 13 to 59% when *KRAS* and *NRAS* codons 12, 13, 59, 61, 117, and 146; *BRAF* codon 600; and *PIK3CA* exon 20 were included. However, more prospective studies

are needed to elucidate the benefit of testing *BRAF* and *PIK3CA* mutations to guide anti-EGFR therapy.

Proximal and distal colorectal cancers differ in terms of clinicopathological features and molecular oncogeneisis.[30–32] *BRAF* mutations are predominantly observed in right-sided colorectal cancers regardless of microsatellite instability status.[31,33] Association of *KRAS* mutations with tumor location remains controversial. Most large studies of more than 1000 colorectal cancers showed consistently higher incidences of *KRAS* codons 12 and 13 mutations in the right-sided colorectal cancers (38–40 *vs* 30–35% similar to 43 *vs* 36% in this study), albeit with a much less substantial difference than that seen in *BRAF* mutations.[33–36] In the current study with broader reportable ranges of the next-generation sequencing assay, right-sided colorectal cancers showed a higher incidence of mutations in *KRAS*, *NRAS*, and *BRAF* genes within the MAPK pathway. The observation that *RAS* mutations occur at a higher frequency in cecal cancers compared with colorectal cancers in other locations has also been reported previously.[34,36] These results were consistent with the gene expression profiles showing activated signature of the MAPK pathway in the right-sided colorectal cancers and the clinical study showing a greater progress-free survival benefit from cetuximab therapy in left-sided colorectal cancer patients with wild-type *KRAS* status.[31,37]

Consistent mutant allele frequencies over a 20-months period in positive control specimens highlighted again the precise quantitative nature of the next-generation sequencing assays.[21] During our validation of this clinical assay, we also showed that mutant allele frequencies of *KRAS* and *BRAF* mutations detected by pyrosequencing and next-generation sequencing were highly concordant, suggesting that next-generation sequencing assays are also accurate in their quantification.[18,38] We have also shown that correlation of the observed mutant allele frequency and the expected mutant allele frequency based on the estimated tumor cellularity may serve as a measure for post-analytic quality assessment.[18,22] The discrepancy between observed and expected mutant allele frequencies may result from tumor heterogeneity, mutant allele-specific imbalance, assay bias and/or inaccurate estimation of tumor cellularity.

Mutations occurring after initiation of the founder clone may be present in only a subclonal population. Whether these mutations could be detected depends on the analytic sensitivity of assays used and the portion of tumor examined. Mutations may be present in a subclonal population when the observed mutant allele frequency is lower than that expected from the tumor cellularity (Table 2). This was confirmed in the current study by examining separate areas of neoplasm. Detection of *KRAS* or *NRAS* mutations in a subclonal population of colorectal cancers may be clinically informative. In colorectal cancers, tumors with 'secondary' *KRAS* or *NRAS* mutations may arise from a small subpopulation present within the original tumor before treatment or as a consequence of continued mutagenesis over the course of anti-EGFR treatment.[39,40] Presence of KRAS-mutant subclones has also shown correlation with worse progression free survival in colorectal cancer patients treated with anti-EGFR therapy.[32]

Because it is quantitative, next-generation sequencing also provides the relative mutant allele frequencies of concomitant mutations. Discrepancy of mutant allele frequency in concomitant mutations is most likely caused by mutant allele-specific imbalance, but it may also indicate that one mutant is present in a subclonal population. In this study, the same *KRAS* mutations were observed in two pairs of invasive neuroendocrine carcinomas and adjacent adenomas, but only invasive tumors showed a *PIK3CA* mutation. This explains only an 8.9% *PIK3CA* mutation in the original sampling of both adenoma and invasive neuroendocrine carcinoma with an overall 61–80% estimated tumor cellularity in case 282. These results also indicate that the invasive neuroendocrine carcinoma is derived from the adenoma after gain of the *PIK3CA* mutation, similar to the stepwise genetic alteration model associated with colorectal tumorigenesis. This model proposes that *PIK3CA* mutations occur after *KRAS* and *BRAF* mutations and, in cooperation with other oncogenes or tumor suppressor genes, drive the clonal evolution from large adenoma to invasive adenocarcinoma.[41]

Next-generation sequencing demonstrates a high analytic sensitivity, broad reportable range of mutation spectrum, capacity for quantitative measurement of mutant allele frequencies, and simultaneous detection of concomitant mutations. Mutant allele frequency and tumor cellularity can be used as a quality assessment measure to predict and identify tumor heterogeneity and mutant allele-specific imbalance. Further studies are warranted to elucidate the clinical and/or biological significance of uncommon mutations, concomitant mutations, mutant allele-specific imbalance, and anti-EGFR resistant mutations detected in minor subclonal populations.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)