Human Pathology (2019) **86**, 12–20

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Human PATHOLOGY

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Original contribution

Clinical validation of coexisting driver mutations in colorectal cancers☆,☆☆

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Received 12 October 2018; revised 13 November 2018; accepted 16 November 2018

Keywords:
Coexisting mutation;
BRAF;
KRAS;
NRAS;
PIK3CA;
Colorectal cancer

Summary Mutational profiling is recommended for selecting targeted therapy and predicting prognosis of metastatic colorectal cancer (CRC). Detection of coexisting mutations within the same pathway, which are usually mutually exclusive, raises the concern for potential laboratory errors. In this retrospective study for quality assessment of a next-generation sequencing assay, we examined *BRAF*, *KRAS*, and *NRAS* genes within the mitogen-activated protein kinase (MAPK) pathway and the *PIK3CA* gene within the phosphatidylinositol 3-kinase (mTOR) pathway in 744 CRC specimens submitted to our clinical diagnostics laboratory. Although coexistence of mutations between the MAPK and mTOR pathways was observed, it rarely occurred within the MAPK pathway. Retrospective quality assessments identified false detection of coexisting activating *KRAS* and *NRAS* mutations in 1 specimen and confirmed 2 activating *KRAS* mutations in 2 specimens and coexisting activating *KRAS* and *NRAS* mutations in 2 specimens, but no coexisting activating *RAS* and *BRAF* mutations. There were 15 CRCs with a kinase-impaired *BRAF* mutation, including 3 with a coexisting activating *KRAS* mutation, which may have therapeutic implications. Multiregional analysis based on different histologic features demonstrated that coexisting *KRAS* and *NRAS* mutations may be present in the same or different tumor populations and showed that invasion of adenomas by synchronous adenocarcinomas of different clonal origin may result in detection of coexisting mutations within the MAPK pathway. In this study, we proposed an operating procedure for clinical validation of unexpected coexisting mutations. Further studies are warranted to elucidate the biological significance and clinical implications of coexisting mutations within the MAPK pathway.

1. Introduction

Anti-EGFR monoclonal antibodies have been approved by the Food and Drug Administration in the United States for targeted therapy of metastatic colorectal cancer (CRC) with wild-type *KRAS* and *NRAS* genes [1,2]. Mutational profiling of these genes is part of the Food and Drug Administration label

☆ Competing interests: The authors declare that they have no competing interests.

☆☆ Funding/Support: This retrospective analysis for quality improvement was supported by 1UM1CA186691-01 from the National Institutes of Health–National Cancer Institute of the United States.

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https://doi.org/10.1016/j.humpath.2018.11.014
0046-8177/

for these drugs and is considered standard of care for patients with metastatic CRC, along with *BRAF* mutational testing for prognostic stratification of CRC and evaluation of Lynch syndrome risk in mismatch repair–deficient CRC [2,3].

KRAS mutations within the mitogen-activated protein kinase (MAPK) pathway promote the progression of small adenomas to large adenomas, whereas *PIK3CA* mutations within the phosphatidylinositol 3-kinase (mTOR) pathway promote the progression of large adenomas to adenocarcinomas [4,5]. Therefore, coexistence of mutations within the MAPK pathway and *PIK3CA* mutations within the mTOR pathway are common in CRC, whereas activating *KRAS*, *NRAS*, and *BRAF* mutations are thought to be mutually exclusive [6,7]. This is also true for genes within the EGFR and MAPK pathway in non–small cell lung cancer [8].

We have previously shown an extremely low incidence (0.1%) of coexisting activating *EGFR* and *KRAS* mutations in non–small cell lung cancer in a clinical diagnostic setting [9]. However, a higher incidence (0.6%-1.5%) has been reported in retrospective research studies [10,11]. Detection of unusual coexisting mutations should raise the concern for potential laboratory errors, which may occur at any point during the preanalytical, analytical, and/or postanalytical phases of testing [12,13]. In this retrospective study for quality assessment of coexisting mutations detected within the MAPK pathway (*KRAS*, *NRAS*, and *BRAF* genes) and/or mTOR pathways (*PIK3CA* gene) in CRC, we propose a standard operating procedure to confirm the presence of coexisting mutations and to evaluate whether these coexisting mutations occur in the same tumor population or in different but physically adjacent tumor populations within the same tissue block.

2. Materials and methods

2.1. Materials

Next-generation sequencing (NGS) results from 744 CRC specimens submitted to the Johns Hopkins Molecular Diagnostics Laboratory between April 2013 and December 2016 were analyzed for mutations within the MAPK and mTOR pathways. DNA was isolated from formalin-fixed, paraffin-embedded tissues, purified, and measured as described previously [14]. The Johns Hopkins Medicine Institutional Review Board granted approval for this study.

2.2. Next-generation sequencing

NGS was conducted using AmpliSeq Cancer Hotspot Panel (v2; Life Technologies, Carlsbad, CA) for targeted multigene amplification as described previously [7,14]. Sequencing data of the targeted genes were analyzed using Torrent Suite (Life Technologies). Mutations were identified and annotated through both Torrent Variant Caller and direct visual inspection of the binary sequence alignment/map file using the Broad Institute's Integrative Genomics Viewer [15]. CRC specimens were analyzed for *BRAF* (NM_004333), *KRAS* (NM_033360), *NRAS* (NM_002524), and *PIK3CA* (NM_006218) genes for clinical reporting. *BRAF* mutations were categorized into 3 classes according to the BRAF kinase activity (class 1, codon 600 mutants with high or intermediate kinase activity; class 2, noncodon 600 mutants with high or intermediate kinase activity; and class 3, low or no kinase activity) [16-18]. The analytic performance characteristics and the reportable ranges have been reported previously in the first 304 CRC specimens [7]. The limit of detection was 2% mutant alleles. An operating procedure was followed to confirm unusual coexisting mutations (Fig. 1). In short, a tissue identification assay was performed to identify potential tissue or DNA mix-up. In the absence of mix-up, it was followed by multiregional analysis by pyrosequencing and NGS to confirm coexisting mutations and to determine if mutations were present in the same or different subpopulations.

2.3. Prediction of clonality in specimens with coexisting mutations

The expected variant allele frequency (VAF) is half of the estimated tumor percentage for a heterozygous mutation. We have previously shown that a lower-than-expected VAF indicates the presence of the mutation in a subpopulation [7]. In this study, coexisting mutations detected in a specimen were predicted to be present in different neoplastic populations when the sum of the 2 observed VAFs was lower than or equivalent to the estimated VAF. In contrast, combined VAFs of coexisting mutations higher than the estimated VAF suggested that both mutations were present in the same population, particularly when the 2 VAFs were concordant with each other and the estimated VAF. Of note, subjective estimation of neoplastic cellularity and presence of mutant allele–specific imbalance (gain of the mutant allele or loss of the wild-type allele) may lead to a false prediction [19,20].

2.4. Multiregional analysis to determine clonality

DNA was retrospectively reisolated from multiple subareas within the originally designated area(s) of specimens with coexisting mutations. Subareas were defined based on physical separation and/or differences in histomorphology of the neoplastic cells, such as adenoma versus adenocarcinoma or low-grade versus high-grade, if present (Fig. 1). DNA was isolated from 3 or more randomly selected subareas if clearly distinctive morphologic features were not identifiable. Detection of coexisting mutations in each subarea with similar VAF ratios was suggestive of a neoplastic population containing both mutations.

2.5. Pyrosequencing

Pyrosequencing was performed as previously described [21]. The limit of detection of pyrosequencing is approximately 5%

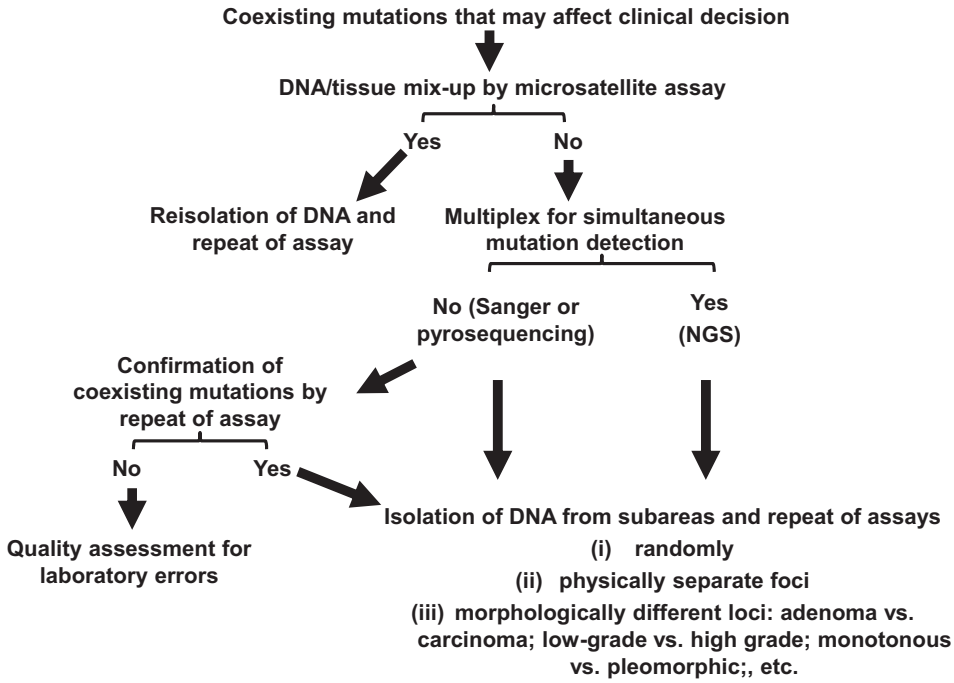

Fig. 1 A proposed operating procedure to evaluate unexpected coexisting mutations in a clinical diagnostics setting.

mutant alleles. The polymerase chain reaction primers were aggcctgctgaaaatgactgaatataa-3′ and 5′-caaagaatggtcctgcaccag taatat-3′ for *KRAS* codons 12/13 mutations, 5′-gactctgaagatg tacctatggtccta-3′ and 5′-cagatctgtatttatttcagtggtacttacct-3′ for *KRAS* codon 146 mutations, 5′-gttcttgctggtgtgaaatgactg-3′ and 5′-cctcacctctatggtgggatcatat-3′ for *NRAS* codons 12/13 mutations, and 5′-caccccaggattcttacagaaaa-3′ and 5′-ttcgcctgtcctcatgtattgg-3′ for *NRAS* codon 61 mutations. The sequencing primer was 5′-cttgtggtagttggagct-3′ for *KRAS* codons 12/13 mutations, 5′-ggaattccttttattgaaacatca-3′ for *KRAS* codon 146 mutations, 5′-ctggtggtggttggagca-3′ for *NRAS* codons 12/13 mutations, and 5′-atactggatacagctgga-3′ for *NRAS* codon 61 mutations.

2.6. Tissue identity

Microsatellite analysis for tissue identification was performed using the *AmpFlSTR* Identifiler kit (Applied Biosystems, Foster City, CA), as described previously [22]. The assay has been clinically validated for detection of post-transplant chimerism. The limit of detection for the minor component is 1% to 5% alleles.

2.7. Statistical analysis

χ^2 Test was performed to calculate *P* values.

3. Results

3.1. Coexisting mutations in CRC

Mutational profiling data of the first 304 CRCs have been reported previously [7]. Current expanded study populations showed a similar mutation rate in each gene (Table 1). Activating mutation of the *BRAF*, *KRAS* and *NRAS* genes were observed

Table 1 Mutational profiling of 744 CRCs

Gene	Incidence of mutation [a]	Incidence of activating mutation
BRAF	86 (12%)	68 (9.1%) [b]
KRAS	353 (47%)	351 (47%) [c]
NRAS	35 (4.7%)	34 (4.6%) [c]
PIK3CA	135 (18%)	90 (12%) [d]

[a] Incidence of mutation in each gene among the first 304 CRCs has been reported previously [7].
[b] Defined as mutations with high kinase activity [16-18].
[c] Defined as mutations involving codons 12, 13, 59, 61, 117, and 146.
[d] Defined as mutations involving codons 542, 545, and 1047.

Table 2 Coexisting mutations in 774 CRCs [a]

Gene [b]	*BRAF*	*KRAS*	*NRAS*	*PIK3CA*	%CM [c]	*P* [d]
BRAF (86)	1 (1.1%)	5 (5.8%)	1 (1.1%)	18 (21%)	28	<.001
KRAS (353)	5 (1.4%)	6 (1.7%)	4 (1.1%)	69 (20%)	22	<.001
NRAS (35)	1 (2.9%)	4 (11%)	0 (0%)	6 (17%)	31	<.001
PIK3CA (135)	18 (13%)	69 (51%)	6 (4.4%)	14 (10%) [e]	69	

[a] Incidence of coexisting mutations among the first 304 CRCs have been reported previously [7].
[b] Numbers in parentheses indicate case number of CRC with a gene-specific mutation.
[c] Percentage of *BRAF*-, *KRAS*-, *NRAS*-, or *PIK3CA*-mutated CRC containing coexisting mutations (CM) within other genes.
[d] Compared with %CM of *PIK3CA*-mutated CRC.
[e] Nine CRCs with 2 *PIK3CA* and 1 *KRAS* mutations, 1 CRC with 2 *PIK3CA* and 1 *BRAF* mutations, 3 CRC with 2 *PIK3CA* mutations, and 1 CRC with 3 *PIK3CA* mutations.

in 68 (9.1%), 351 (47%), and 34 (4.6%) of CRCs, respectively. Coexisting mutations within *BRAF*, *KRAS*, *NRAS*, and *PIK3CA* genes were observed in 114 (15%) CRCs (Table 2). These included 21 (2.8%) CRCs with coexisting mutations within the same gene (1 with 2 *BRAF* mutations, 6 with 2 *KRAS* mutations, 13 with 2 *PIK3CA* mutations, and 1 with 3 *PIK3CA* mutations) and 103 (14%) CRCs with coexisting mutations in different genes. Among the 13 CRCs with 2 *PIK3CA* mutations, coexistence with a *KRAS* or *BRAF* mutation was seen in 10 CRCs. *PIK3CA*-mutated CRC showed a significantly higher incidence of coexisting mutations (Table 2). The incidence of coexisting *PIK3CA* mutations was not significantly different among *BRAF*-, *KRAS*-, or *NRAS*-mutated CRCs (21%, 20%, and 17%, respectively).

3.2. Coexisting mutations within the *MAPK* pathway

Although coexisting mutations among genes in the mTOR pathway and the MAPK pathway were common, coexisting activating mutations within the MAPK pathway were rare and therefore raised the concern for potential laboratory errors (Table 3). Microsatellite-based identity testing showed no minor peaks to indicate a mixture of DNA from 2 or more individuals in all specimens listed in Table 3. Multiregional analysis was performed using pyrosequencing and NGS to evaluate whether the coexisting mutations occurred in the same or different tumor populations.

3.3. Coexisting *BRAF* and *RAS* mutations

NGS detected 67 CRCs with a class 1 *BRAF* p.V600E mutation, 2 with a class 2 *BRAF* mutation (p.G469A and p.G469R), 15 with a class 3 *BRAF* mutation (9 involving codon 594), 1 with p.G606R of unknown kinase activity, and 1 with coexistence of activating p.V600E mutation (class 1) and kinase-impaired p.D594G mutation (class 3). Among the 6 *BRAF* mutations detected in *KRAS*- or *NRAS*-mutated CRC, 4 were kinase impaired (*BRAF* p.G466E plus *KRAS* p.Q22K of uncertain activating status, *BRAF* p.Y472C plus *KRAS* p.G12 V, *BRAF* p.D594G plus *KRAS* p.A59E, and *BRAF* p.D594G plus *KRAS* p.G12D), and 1 was a class 2 *BRAF* mutation with intermediate kinase activity (*BRAF* p.G469R plus *NRAS* p.G12D) [16-18,23]. The remaining one with p.V600E was seen in CRC0145 with *KRAS* p.G15S of uncertain activating status. Thus, there were no cases with coexisting activating *KRAS* mutation and *BRAF* mutation with high kinase activity.

3.4. Dual activating *KRAS* mutations

Two activating *KRAS* mutations were seen in 3 (0.4%) of 744 CRCs (Table 3). There were no doublet-activating *NRAS*

Table 3 Coexisting activating mutations within the MAPK pathway in CRCs

Case	Tumor %	Mut 1 (VAF%)	Mut 2 (VAF%)	Clonality (VAFp/MRA) [a]
KRAS + *KRAS*				
CRC0256 (Re)	61-80	*KRAS* p.G12A (17)	*KRAS* p.G13D (17)	Different/different
CRC0340 (Bx)	61-80	*KRAS* p.G13C (32)	*KRAS* p.A146V (30)	Same/NE
CRC0449 (Re)	61-80	*KRAS* p.G13 V (14)	*KRAS* p.G13D (21)	Different/different
KRAS + *NRAS*				
CRC0068 (Re)	61-80	*NRAS p.Q61K* (14)	*KRAS* p.G12D (15)	Different/different
CRC0125 (Re)	61-80	*NRAS* p.Q61R (15)	*KRAS* p.A146T (57)	Same/no CM [b]
CRC0202 (Re)	61-80	*NRAS* p.G13C (36)	*KRAS* p.G12 V (5.2)	Different/same [c]

Abbreviations: Bx, biopsy; CM, coexisting mutation; ;NE, not evaluated; Re, resection VAF, variant allele frequency;.
[a] VAFp: prediction of clonality by VAF. MRA: evaluation of clonality by multiregional analysis.
[b] Only *KRAS* p.A146T was detected in the repeated NGS assay and multiregional analysis.
[c] Both *KRAS* and *NRAS* mutations in adenoma, but only *NRAS* in invasive adenocarcinoma.

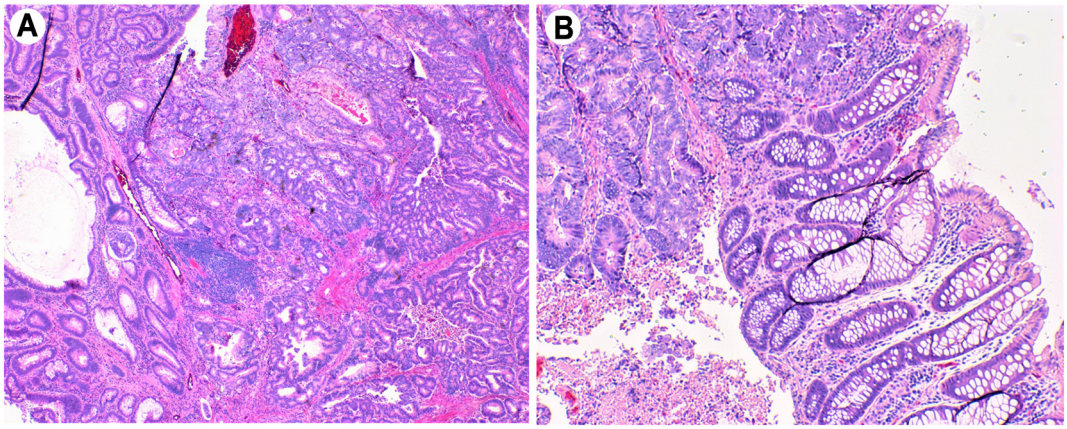

Fig. 2 Invasive adenocarcinoma and adjacent adenoma harboring different *KRAS* mutations. In CRC0256, the invasive adenocarcinoma has expanded to abut the adenoma (A) and invade the normal epithelium (B). Multiregional analysis by pyrosequencing and NGS showed *KRAS* p.G13D in the adenoma (subareas 6 and 7) and *KRAS* p.G12A in the adenocarcinoma (subareas 1-5; not shown). The original magnifications of the E ;hematoxylin and eosin–stained slides are ×100.

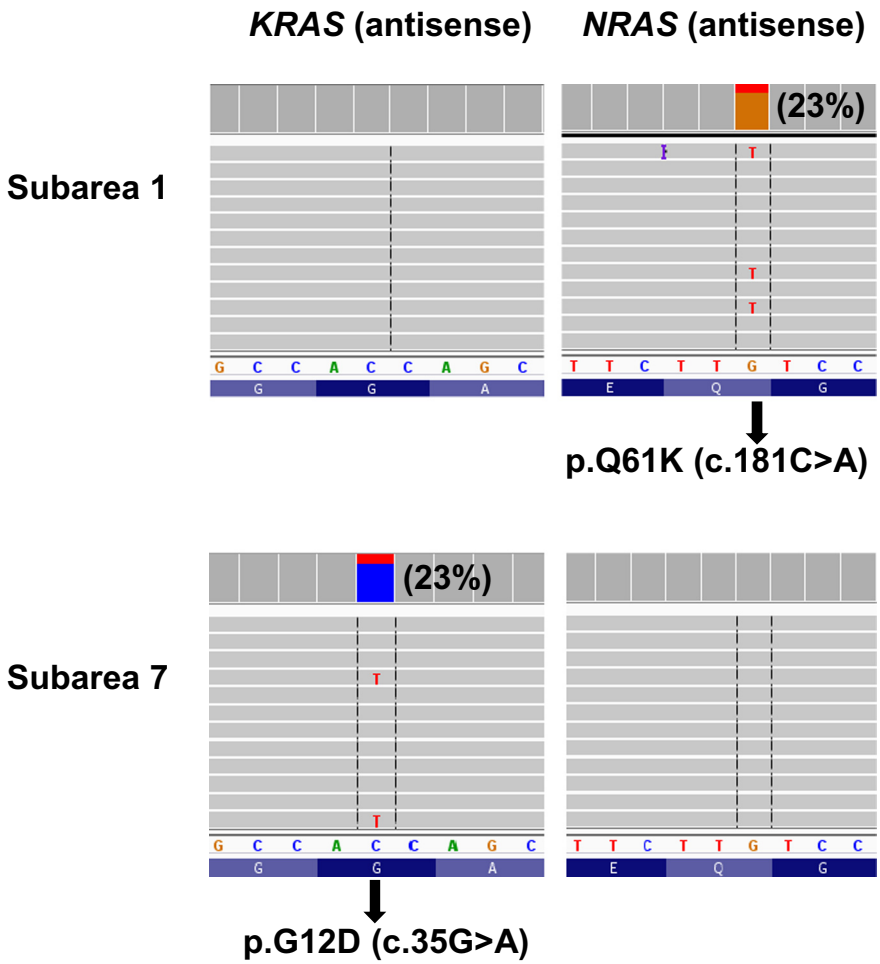

Fig. 3 Two adjacent invasive adenocarcinomas harboring different *RAS* mutations. In specimen CRC0068, pyrosequencing showed *NRAS* p.Q61K in subareas 1 to 5 with an infiltrative growth pattern and *KRAS* p.G12D in subareas 6 to 8 with a nodular growth pattern. These were also confirmed by NGS analysis of the subareas 1 and 7. Percentage in parentheses indicates VAF.

mutations. Review of hematoxylin and eosin slides from CRC0256 and CRC0449 showed an invasive adenocarcinoma component and an adjacent adenoma component within the originally designated tumor areas isolated for testing (Fig. 2). Multiregional analysis of CRC0256 and CRC0449 showed a different *KRAS* mutations within the adenocarcinoma in comparison to the adjacent adenoma, with the sum of the 2 observed VAFs equivalent to the estimated VAF based on tumor cellularity (Table 2). Three specimens showed coexistence of an activating *KRAS* mutation and a *KRAS* mutation of unknown activating status (p.G12D plus p.Q22K, p.G12 V plus p.V14I, and p.G12D plus p.G60S).

3.5. Coexisting activating *KRAS* and *NRAS* mutations

One specimen showed coexistence of activating *KRAS* p.G12D mutation and *NRAS* p.G60E of unknown activating status. Coexisting activating *KRAS* and *NRAS* mutations were seen in 3 CRCs (Table 3). In CRC0068, which had coexisting *KRAS* p.G12D and *NRAS* p.Q61K mutations, the sum of the VAFs predicted that each mutation was present in a different population. This was confirmed by multiregional analysis of the invasive adenocarcinoma (Fig. 3).

Coexisting *KRAS* p.A146T and *NRAS* p.Q61R mutations were detected in CRC0125. Pyrosequencing, however, showed only the *KRAS* mutation from 5 subareas. NGS repeated on the originally examined DNA sample and on DNA samples from 2 subareas also showed only *KRAS* p.A146T. The results indicate that the *NRAS* mutation was not present in this CRC. False detection of *NRAS* mutation in the original NGS assay was most likely the result of contamination with an *NRAS* mutant tumor during library preparation or the sequencing processes.

In CRC202, which had coexisting *KRAS p.G12 V* and *NRAS p.G13C* mutations, the originally examined DNA was isolated from a designated area containing a dominant invasive adenocarcinoma component and an adjacent minor adenoma component. NGS detected a 5.2% *KRAS* mutation and a 36% *NRAS* mutation in the context of 61% to 80% estimated neoplastic cellularity. Multiregional analysis by pyrosequencing showed only the *NRAS* mutation in 3 adenocarcinoma subareas but both *KRAS* and *NRAS* mutations in a small adenoma subarea. NGS analysis of the adenoma subarea showed a 34% *KRAS* p.G12 V and a 37% *NRAS* p.G13C in a context of 61% to 80% estimated neoplastic cellularity, suggesting that *KRAS* and *NRAS* mutations were present in the same adenoma population (Fig. 4). Pyrosequencing of 2 additional adenoma subareas, which were not included in the originally designated

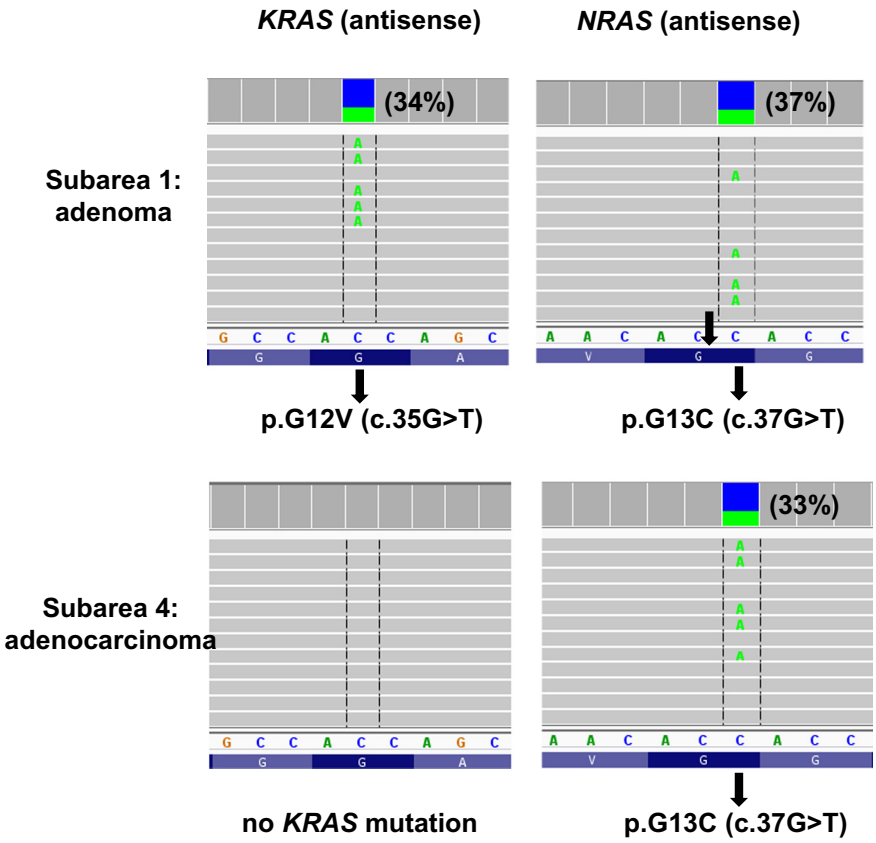

Fig. 4 Coexistence of *KRAS* and *NRAS* mutations in the adenoma, but only *NRAS* mutation in the adenocarcinoma. In CRC202, pyrosequencing showed coexistence of *KRAS* and *NRAS* mutations in a small adenoma subarea (subarea 1), but only the *NRAS* mutation in 3 adenocarcinoma subareas (subareas 2-4). These were also confirmed by NGS analysis of subareas 1 and 4. Percentage in parentheses indicates VAF.

area for examination and physically separated from the invasive adenocarcinoma in a 2-dimentional section, also showed both *RAS* mutations.

4. Discussion

Coexisting mutations within the same signal transduction pathway are often mutually exclusive [6-8,24]. Observation of unexpected coexisting mutations raises the concern for potential laboratory errors, which may occur during any step of preanalytical, analytical, or postanalytical phases. This includes contamination of analytes (tissues, DNA, or polymerase chain reaction products) [12,13]. Swapping of analytes or data files can also lead to the false detection of coexisting mutations, especially when simplex assays, such as Sanger sequencing or pyrosequencing, are applied to retrospective large cohort studies.

We propose an operating procedure to validate coexisting mutations (Fig. 1). When unusual coexisting mutations are observed, previously isolated DNA is examined by microsatellite analysis to confirm tissue identity. If coexisting mutations are detected by a simplex assay, this is followed by either repeating the original assay or using an alternative assay to confirm results. Simultaneous mutational profiling by multiplex assays, such as NGS, may reduce the false detection of coexisting mutations caused by swapping of analytes and/or data files. In this retrospective study, we showed a false detection of coexisting *KRAS* and *NRAS* mutations in one specimen. In the presence of coexisting mutations that may affect clinical decision making, multiregional analysis based on histomorphology not only confirms coexistence of mutations but also determines whether these mutations are present in the same or different populations of tumor cells.

BRAF mutations can be categorized according to their kinase activity [16,25]. Coexistence of activating *RAS* mutations was seen in 3 of 15 CRCs with a kinase-impaired *BRAF* mutation in this study, supporting the reported interaction of oncogenic RAS proteins and kinase-impaired BRAF leading to hyperactivation of the CRAF/MEK/ERK cascade [17,18]. Preclinical studies suggest that tumors with coexisting *RAS* and kinase-impaired *BRAF* mutations may be sensitive to MEK or ERK inhibitors, and tumors with a kinase-impaired *BRAF* mutation and a wild-type *RAS* are predicted to be sensitive to anti-EGFR therapy [17,18]. Response to anti-EGFR therapy in CRC patients with kinase-impaired *BRAF* mutation and wild-type *RAS* has also been reported [6,17].

Coexistence of activating *RAS* mutation and *BRAF* p.V600E was not observed in 744 CRCs and in 1006 lung cancers in our clinical laboratory [9,13]. These findings were consistent with several previous reports with 500 to 1500 white or Asian patients examined for both *KRAS* and *BRAF* mutation in each study. Only 1 patient with coexisting activating *RAS* and *BRAF* p.V600E mutations was detected in a total of more than 200 CRCs with class 1 *BRAF* mutations (mainly p.V600E) [6,25-30]. In another report with 4411 CRC patients, *KRAS* mutations within codons 12 and 13 were not seen in 480 *BRAF* p.V600E-muated CRCs. However, coexistence of activating *KRAS* mutation and *BRAF* mutation involving codon 600 or 601 was reported in 9 (32%) of 28 LN metastases of CRC [31], and in 6 (0.5%) of 1261 CRCs or 6 (3.3%) of 181 *BRAF*-mutated CRCs [32]. Rare cases with coexistence of an activating *RAS* mutation and *BRAF* p.V600E have also been reported [33-36].

KRAS and *NRAS* mutations are also expected to be mutually exclusive, although preclinical data have shown divergent oncogenic properties of the *KRAS* p.G12D and *NRAS* p.G12D mutations [37]. Coexisting *KRAS* and *NRAS* mutations were not seen in 2 studies with approximately 500 to 700 CRC patients [6,38] but were reported in 11 (0.9%) of 1294 European CRC patients [26] and in 1 (0.2%) of 621 and 8 (0.7%) of 1110 Chinese CRC patients [28,29]. In this study, the coexistence of activating *KRAS* and *NRAS* mutation was initially reported in 3 of 744 CRCs. Retrospective quality assessment confirmed false detection of coexistence in one of these specimens. Multiregional analysis revealed presence of *KRAS* and *NRAS* in separate invasive adenocarcinoma components submitted within the same tissue blocks (CRC0068) and presence of both *KRAS* and *NRAS* mutations in an adenoma component (CRC0202).

Dual *KRAS* mutations have also been reported in 4 (0.5%) of 747 European CRC patients and 8 (0.7%) of 1110 Chinese CRC patients [6,29]. Whether the presence of 2 *KRAS* mutations was seen in the same or different tumor populations is not reported. In this study, NGS detected 3 (0.4%) of 744 CRCs with coexistence of 2 activating *KRAS* mutations. Multiregional analysis confirmed different *KRAS* mutations within the invasive adenocarcinoma component and the adjacent adenoma component in CRC0256 and CRC0449. Coexistence of 2 activating *KRAS* mutations within an individual adenocarcinoma component was not seen in 744 CRCs of this study. Analysis of the entire NGS panel revealed an *APC* mutation (p.R1450* in CRC0265 and p.P1443fs in CRC0449, data not shown) in the adenoma but not the adenocarcinoma, indicating that CRC0256 and CRC0449 each contained a collisional adenoma and adenocarcinoma originating from different ancestral clones. The adenocarcinoma may have expanded laterally and upward to invade a synchronous adenoma. In the absence of molecular profiling, the histomorphology could have been simply interpreted as colonic adenocarcinoma arising from an adenoma.

Invasion or abutting of a colonic adenocarcinoma into an adenoma of different clonal origin may have clinical implications. In the current guidelines for standard of care of metastatic CRC published by the College of American Pathologists and the Association of Molecular Pathology, *KRAS*, *NRAS*, and *BRAF* are the only 3 genes recommended for mutational profiling [3]. In the multistep model for colorectal tumorigenesis [4,5], *KRAS*, *NRAS*, and *BRAF* mutations, which are all part of the MAPK pathway, represent trunk (initiating) drivers to promote progression from small adenoma to large adenoma, a step before the formation of the founder cell of

adenocarcinomas. These trunk drivers should be present in the invasive adenocarcinomas and their adjacent precursor adenomas. Therefore, inclusion of the precursor adenoma for mutational profiling of trunk drivers, such as the *KRAS* mutational status, is expected to be concordant with those from the invasive adenocarcinoma component. However, 2 or more colonic polyps may develop in close proximity, which may lead an adenocarcinomas to invade upward and collide with a neighboring unrelated adenomas. Endoscopic biopsy of a superficial lesion may therefore contain a minor invasive adenocarcinoma component insufficient for molecular profiling and a dominant adenoma component originating from a different ancestral clone carrying different trunk driver mutations.

We propose an operating procedure for clinical validation of coexisting activating mutations of the *BRAF*, *KRAS*, and *NRAS* genes within the MAPK pathway, which are expected to be mutually exclusive in colorectal tumorigenesis. Multiregional analysis according to this operating procedure confirmed a rare incidence of coexisting activating *RAS* mutations, determined their presence in the same or different tumor populations, and identified invasion of a synchronous adenoma by a collisional adenocarcinoma of different clonal original. Further studies are warranted to elucidate the biological significance and clinical implications of coexisting activating mutations within the MAPK pathway.

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