Published OnlineFirst January 26, 2010; DOI: 10.1158/1078-0432.CCR-09-2446

Human Cancer Biology

Clinical Cancer Research

Prevalence and Heterogeneity of *KRAS, BRAF*, and *PIK3CA* Mutations in Primary Colorectal Adenocarcinomas and Their Corresponding Metastases

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Abstract

Purpose: Epidermal growth factor receptor (EGFR) antibody therapy is established in patients with wild-type *KRAS* colorectal carcinoma; however, up to 50% of these patients do not respond to this therapy. To identify the possible causes of this therapy failure, we searched for mutations in different EGFR-dependent signaling proteins and analyzed their distribution patterns in primary tumors and corresponding metastases.

Experimental Design: Tumor tissues, macrodissected from tumor centers, invasion fronts (n = 100), lymph nodes (n = 55), and distant metastases (n = 20), respectively, were subjected to DNA extraction and mutation analysis of *KRAS, BRAF*, and *PIK3CA*.

Results: Activating mutations were detected in 41% (*KRAS*), 7% (*BRAF*), and 21% (*PIK3CA*) of the primary tumors. By comparing tumor centers and invasion fronts, the intratumoral heterogeneity of *KRAS, BRAF*, and *PIK3CA* mutations was observed in 8%, 1%, and 5% of primary tumors, respectively. Heterogeneity between primary tumors and lymph node metastases was found in 31% (*KRAS*), 4% (*BRAF*), and 13% (*PIK3CA*) of the cases. Heterogeneity between primary tumors and distant metastases was present in two patients (10%) for *KRAS* and one patient for *PIK3CA* (5%), but not for *BRAF*. Discordant results between primary tumors and metastases could markedly be reduced by testing the additional tumor samples.

Conclusions: Failure of EGFR antibody therapy in patients with wild-type *KRAS* colorectal cancer may result from activating *BRAF* or *PIK3CA* mutations and false-negative sequencing results caused by intratumoral heterogeneity. Due to the particularly high rates of heterogeneity between primary tumors and lymph node metastases, the latter are least suitable for diagnostic mutation analysis. *Clin Cancer Res; 16(3); 790–9. ©2010 AACR.*

Based on the increasing knowledge of cancer cell–specific signaling pathways, targeted therapy was introduced into oncologic concepts and now represents an important approach in clinical cancer therapy. With regard to colorectal cancer, the therapeutic benefit of epidermal growth factor receptor (EGFR)–specific monoclonal antibodies such as cetuximab and panitumumab has been established in various studies (1–8). Interestingly, in search of predictive molecular markers, no correlation could be observed between the expression levels of EGFR and therapeutic success (1–3). Surprisingly, even patients with tumors apparently lacking EGFR expression responded to antibody therapy in up to 25% of the cases (9–11). This lack of correlation could at least partially be explained by sampling errors as most colorectal carcinomas express EGFR in a heterogenous way.

More importantly, however, it has been shown that the failure of EGFR antibody therapy may be caused by activating mutations of EGFR-dependent signaling molecules. Two main EGFR-dependent signaling pathways are the KRAS-BRAF and the PIK3CA-PTEN-AKT pathways, which regulate cell proliferation, survival, cell growth, apoptosis resistance, invasion, and migration (12, 13). In various clinical studies, including cetuximab or panitumumab in second- or third-line therapy, only patients with wild-type *KRAS* responded to therapy (up to 50% of all patients), whereas patients with tumors exhibiting *KRAS* mutations had a response rate between 0% and 6% (14–19). In a first-line study, administering cetuximab in addition to a FOLFOX-4 regimen, patients with *KRAS* wild-type tumors showed a better response and a significant increase in both progression-free and overall survival compared with patients with tumors carrying *KRAS* mutations (20). In another first-line study, the benefit of a combination

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doi: 10.1158/1078-0432.CCR-09-2446

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Translational Relevance

Recent studies on colorectal cancer have shown that tumors with KRAS mutations are resistant to antibody-based epidermal growth factor receptor (EGFR)–targeted therapy. However, ~50% of *KRAS* wild-type patients also do not benefit from these studies. To identify the possible causes for this therapy failure, we simultaneously investigated the mutations in the EGFR-downstream genes *KRAS*, *BRAF*, and *PIK3CA*. As a possible cause of false-negative testing results, the prevalence of intratumoral genetic heterogeneity was investigated, as well as the heterogeneity between primary tumor and corresponding metastases. According to our results, failure of EGFR therapy in *KRAS* wild-type patients may at least partly be explained by activating *BRAF* and *PIK3CA* mutations and false-negative sequencing results caused by intratumoral heterogeneity. Lymph node metastases revealed to be the least suitable tumor specimen for mutation analysis. We suggest including the mutation analysis of all the three EGFR downstream genes as predictive markers in further prospective clinical trials on EGFR-directed therapy.

therapy with FOLFIRI and cetuximab was also restricted to patients with *KRAS*-wild-type tumors (21). Similarly, as shown for *KRAS* mutations, mutations of other EGFR-dependent signaling molecules such as BRAF and PIK3CA may confer resistance to EGFR-specific antibody therapy in colorectal cancer as well. Thus, in a series of 113 patients receiving cetuximab or panitumumab, none of the 11 patients with tumors carrying *BRAF* mutations in a wild-type *KRAS* background responded to therapy (22). Accordingly, in two other studies, comprising in total 142 patients with metastatic colorectal carcinomas, cetuximab failed to exhibit any significant effect in patients with tumors displaying *PIK3CA* mutations in the background of wild-type *KRAS* (n = 19; refs. 23, 24).

The prevalence of *KRAS*, *BRAF*, and *PIK3CA* mutations, respectively, has recently been investigated. In population-based studies, 32% to 37% of the patients exhibited activating *KRAS* mutations (25–28), 10% to 17% showed activating *BRAF* mutations (27–29), and *PIK3CA* mutations were observed in 15% of the cases (30). Almost all of the mutations affected codons 12 and 13 of *KRAS*, codon 600 of *BRAF*, as well as exons 9 and 20 of *PIK3CA*.

Activating mutations of *KRAS*, *BRAF*, and/or *PIK3CA* can at least in part explain the lack of response to EGFR-targeted antibodies. However, even in patients with wild-type *KRAS*, *BRAF*, and *PIK3CA*, the response rate was not more than 40% to 60%. One explanation could be that due to intratumoral heterogeneity and consecutive sampling errors, some patients may erroneously be tested as "wild-type." Indeed, heterogeneous distribution of *KRAS* mutations within primary colorectal carcinomas could be shown at least in a few patients (31–33). The heterogeneity of *KRAS* mutations has also been found by comparing primary tumors with lymph node and/or distant metastases, respectively (32, 34). This, however, could not be confirmed by others (35–37). In contrast to KRAS, the heterogeneity of *BRAF* and *PIK3CA* mutations has not been investigated in colorectal cancer thus far.

Given the increasing role of *KRAS*, *BRAF*, and *PIK3CA* mutations as predictive biomarkers for EGFR-targeted therapy and the preliminary and contradictory data about the heterogeneous distribution of such mutations in primary tumors and metastases, here, we systematically investigated the incidence and heterogeneity of *KRAS*, *BRAF*, and *PIK3CA* mutations in primary tumors, lymph node metastases, and distant metastases of 100 colorectal carcinomas. The methods used in the present study detect all mutations thus far published in the context of EGFR-directed therapy.

Materials and Methods

A series of 100 patients with colorectal carcinomas (56 males and 44 females; mean age, 65 y; range, 18-94 y) was derived from the files of the Institute of Pathology and the Department of General, Visceral, and Pediatric Surgery of the University of Duesseldorf. In all cases, single samples were taken from both tumor centers and invasion fronts. Additional lymph node metastases were available for 55 patients and distant metastases for 20 patients, respectively. In the case of multiple lymph node and/or distant metastases, one single metastasis of each location was investigated. When subsequent mutation analyses (see below) revealed heterogeneity either within a given primary tumor or by comparing primary tumors with respective lymph node or distant metastases, as far as available, up to four additional tumor samples either of the respective primary tumors or of additional metastases were collected and analyzed.

Tumor DNA preparation. Tumor tissues were marked on standard H&E-stained histologic slides. Afterwards, unstained serial sections of tumor tissues were mounted onto glass slides and macrodissected for DNA extraction. Every macrodissected tumor sample was cross-checked confirming that the percentage of tumor tissue was at least 80%. The extracted tumor cells were dissolved in a total volume of 190 μL digestion buffer (DNA tissue mini kit, Qiagen) and were treated with proteinase K overnight at 56°C. DNA purification was achieved using a nucleic acid robot device (BIO 101, Qiagen).

Sequence analysis. PCR amplification was done in a total volume of 20 μL containing 20 ng genomic DNA, 0.2 mmol/L deoxynucleotide triphosphate, 0.5 units of Taq polymerase (HotStar Taq, QIAGEN), and primers as specified in Table 1. Cycle sequencing analysis of PCR fragments was done with the BigDye Terminator system (PE Biosystems) using amplification primers for bidirectional

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Table 1. List of primers used in this study

Cycle sequencing			
KRAS exon 2	Fwd_5′-AGGCCTGCTGAAAATGACTGAA-3′	Rev_5′-AAAGAATGGTCCTGCACCAG-3′	
BRAF exon 15	Fwd_5′-TGCTTGCTCTGATAGGAAAATG-3′	Rev_5′-AGCCTCAATTCTTACCATCCA-3′	
PIK3CA exon 9	Fwd_5′-GACAAAGAACAGCTCAAAGCA-3′	Rev_5′-ACATGCTGAGATCAGCCAAA-3′	
PIK3CA exon 20	Fwd_5′-ATGATGCTTGGCTCTGGAAT-3′	Rev_5′-GCATGCTGTTTAATTGTGTGG-3′	
Pyrosequencing:			
BRAF exon 15	Fwd: TGCTTGCTCTGATAGGA-AAATGA	Rev: Bio-CAAAATGGATCCAG-ACAACTGTTC	Seq: GGTGATTTTGGTCTAGC.
PIK3CA exon 9	Fwd: Bio-AAGCAATTTCTACA-CGAGATCCT	Rev: TAGCACTTACCTGTGAC-TCCATAG	Seq: AGATCCAATCCATTTTT-GTTGTC
PIK3CA exon 20	Fwd: Bio-TGACTCCATAGAA-AATCTTT	Rev: GAGCAAGAGGCTTTGGA-GTATTT	Seq: GTTGTCCAGCCACCA

NOTE: Primer sequences used for PCR amplification and sequencing. Bidirectional cycle sequencing was done using amplification primers, whereas for pyrosequencing, an internal sequencing primer (*seq*) was used to analyze the biotinylated (*Bio*) DNA strand.

sequencing. The reaction products were analyzed on an ABI PRISM 3700 sequencer (PE Biosystems).

Pyrosequencing. To reevaluate results obtained by standard cycle sequencing as described above, pyrosequencing, which is reported to represent a highly sensitive approach for KRAS mutation detection (38), was used as second independent method. Primers for *KRAS* analysis were used as described in ref. (38). For *BRAF* and *PIK3CA*, primers were designed using the "Pyrosequencing Assay Design Software" (Biotage AB) as summarized in Table 1.

The determination of base composition and mutational status was done using a PyroMarkTMQ24 device together with the appropriate software tools. The cutoff value, discriminating between a mutation and the wild-type sequence, was assigned to 15% mutant allele. This value equals to the sensitivity of standard cycle sequencing approaches, which was determined by dilution experiments mixing wild-type and mutant DNA derived from tumor cell lines (data not shown).

Statistical analysis. To test for correlations between the histopathologic parameters and the incidence of a respective mutation, we used the Fisher's exact test and, whenever appropriate, the χ^2 test. Statistical computations were done with the statistical package SPSS. The level of significance was set at $P < 0.05$.

Results

KRAS. By analyzing one representative tumor sample of tumor centers and invasion fronts, respectively, *KRAS* mutations in codon 12 or 13 were found in 41 (41%) of the primary colorectal carcinomas (Table 2), consistent with mutation frequencies reported in the literature (25–28). In 33 (33%) of all tumors (80% of tumors with *KRAS* mutations), *KRAS* mutations were simultaneously detected at tumor centers and corresponding invasion fronts (Fig. 1A), whereas in eight cases (8% of all tumors and 20% of tumors with *KRAS* mutations), intratumoral heterogeneity became evident. Thus, in six tumors, mutations were found at tumor centers but not at corresponding invasion fronts, whereas two tumors showed an inverse pattern (Fig. 1A). Interestingly, this genetic mosaicism was only observed in Unio Internationale Contra Cancrum (UICC) stage II and stage III, but not in stage IV tumors (Table 2).

In 55 cases, primary tumors and corresponding lymph node metastases could be compared (Fig. 1A). By analyzing one arbitrarily selected single lymph node metastasis per patient, KRAS mutations were detected in at least one tumor location (primary tumor and/or lymph node metastasis) of 31 patients (56%). Fourteen patients (25% of the 55 cases and 45% of tumors with *KRAS* mutations) showed mutated *KRAS* simultaneously in primary tumors and corresponding lymph node metastases. In 15 patients (27% of the 55 cases and 48% of tumors with *KRAS* mutations), however, *KRAS* mutations were exclusively restricted to primary tumors and in 2 patients (4% of the 55 cases and 6% of tumors with *KRAS* mutations) to lymph node metastases. Overall, discordant results between primary tumors and lymph node metastases were observed in 17 patients (31% of the 55 cases and 55% of tumors with *KRAS* mutations).

Due to this high degree of heterogeneity of mutated *KRAS* between primary tumors and lymph node metastases, as observed under routine diagnostic tissue collection conditions (testing one single tumor sample of a given location), additional tumor samples of these patients were analyzed. In cases with mutated *KRAS* in lymph node metastasis, but wild-type *KRAS* in corresponding primary tumors ($n = 2$), two additional samples from tumor centers and invasion fronts each were tested. In both cases, *KRAS* mutations initially found in the lymph node

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Table 2. Prevalence and heterogeneity of *KRAS, BRAF*, and *PIK3CA* mutations

	n	*KRAS*	*BRAF*	*PIK3CA*
		Prevalence/heterogeneity (%)	Prevalence/heterogeneity (%)	Prevalence/heterogeneity (%)
All tumors	100	41/8	7/1	21/5
Stage I/II	40	28/10	5/0	15/3
Stage III	40	53/10	10/3	28/8
Stage IV	20	45/0	5/0	20/5

NOTE: Prevalence and heterogeneity of *KRAS*, *BRAF*, and *PIK3CA* mutations in primary tumors, as determined by analyzing and comparing samples from both tumor centers and invasion fronts. Data are stratified according to UICC tumor stage.

metastases, thereby, could only be detected in at least one sample of the primary tumor (Table 3). For patients initially showing *KRAS* mutations exclusively in primary tumors, but not in corresponding lymph node metastases (n = 15), as far as available (n = 10 cases), further lymph node metastases were analyzed. In 6 of these 10 cases, *KRAS* mutations were found in either one or two additionally analyzed lymph node metastases (Table 3). In the other four cases, such as in the lymph node metastasis tested initially, all additionally analyzed lymph node metastases exhibited wild-type *KRAS*.

Comparing 20 cases of primary tumors and corresponding distant metastases under routine diagnostic tissue collection conditions (testing one single tumor sample of a given location), discordant results for *KRAS* mutations were found in 2 (10%) patients (Fig. 1A). In one of these patients, the mutation was exclusively restricted to the primary tumor; in the other patient, it was exclusively restricted to the distant metastasis. In the latter case, the KRAS mutation could finally also be detected in the primary tumor, when additional tumor samples were tested. In the first case, the discordant result could not be resolved, as no more tissue samples of the distant metastasis were available.

In terms of the exact positions of *KRAS* mutations identified, 68% of these mutations affected codon 12, whereas 32% affected codon 13 (Fig. 2). For a given patient, *KRAS* mutations in primary tumors, lymph node metastases,

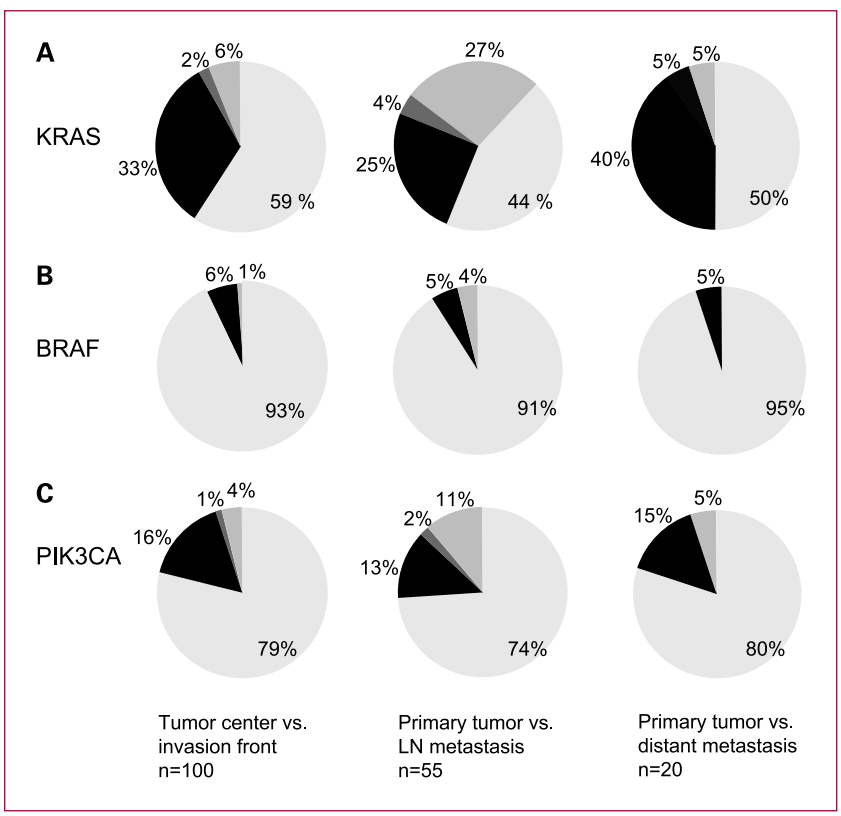

Fig. 1. Heterogeneous distribution of *KRAS, BRAF*, and *PIK3CA* mutations in primary tumors, lymph node, and distant metastases of colorectal cancer. A, mutation status of *KRAS* in corresponding samples of tumor centers and invasion fronts. White, wild-type status in both samples; black, concordant mutations in both samples; dark gray, discordant results showing wild-type *KRAS* at the tumor center and *KRAS* mutations at the invasion front; light gray, discordant results showing *KRAS* mutations at the tumor center and wild-type *KRAS* at the invasion front. The mutation status of *KRAS* in corresponding samples of primary tumor and lymph node or distant metastases, respectively. White, wild-type status in both samples; black, concordant mutations in both samples; dark gray, discordant results showing wild-type status in the primary tumors and *KRAS* mutations in the lymph node or distant metastases; light gray, discordant results showing *KRAS* mutations at the primary tumor and wild-type status in the lymph node or distant metastases. B, mutation status of *BRAF* in corresponding samples of tumor centers and invasion fronts, as described above. C, mutation status of *PIK3CA* in corresponding samples of tumor centers and invasion fronts, as described above.

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Table 3. Detailed analysis of patients showing heterogeneity in terms of *KRAS*, *BRAF*, and *PIK3CA* mutations

Patient ID number	C1	C2	C3	F1	F2	F3	L1	L2	L3
KRAS									
25	Wt	c.35G>A	c.35G>A	Wt	c.35G>A	c.35G>A	c.35G>A		
80	Wt	Wt	c.35G>A	Wt	Wt	Wt	c.35G>A		
30	c.38G>A			c.38G>A			Wt	c.38G>A	
33	c.34G>T			c.34G>T			Wt	Wt	Wt
36	c.38G>A			Wt			Wt	c.38G>A	Wt
49	c.35G>A			c.35G>A			Wt	c.35G>A	c.35G>A
51	c.35G>T			c.35G>T			Wt	Wt	
73	c.38G>A			Wt			Wt	Wt	Wt
75	c.35G>A			c.35G>A			Wt	c.35G>A	c.35G>A
77	c.35G>T			c.35G>T			Wt	Wt	
92	c.35G>T			c.35G>T			Wt	c.35G>T	
99	c.35G>A			c.35G>A			Wt	c.35G>A	c.35G>A
BRAF									
98	c.1799T>A			c.1799T>A			Wt	c.1799T>A	c.1799T>A
PIK3CA									
23	Wt	Wt	c.3140A>T	Wt	Wt	Wt	c.3140A>T		
33	c.1633G>A			Wt			Wt	Wt	Wt
36	c.1633G>A			Wt			Wt	c.1633G>A	Wt
50	c.3140A>G			c.3140A>G			Wt	Wt	
53	c.1633G>A			Wt			Wt	c.1633G>A	c.1633G>A
73	c.1636C>A			c.1636C>A			Wt	c.1636C>A	Wt
91	c.1636C>A			c.1636C>A			Wt	Wt	Wt

NOTE: Detailed analysis of patients showing heterogeneity in terms of *KRAS*, *BRAF*, and *PIK3CA* mutations, respectively, between primary tumor and corresponding lymph node metastasis. In cases, in which a mutation was initially detected in the lymph node metastasis, but neither in the center (*C1*) nor at the invasion front (*F1*) of the respective primary tumor (*KRAS*: $n = 2$; *PIK3CA*: $n = 1$), four additional areas of the primary tumor [2× invasion front (*F2* and *F3*) and 2× tumor center (*C2* and *C3*)] were analyzed. In cases, in which the mutation was initially detected only in the primary tumor, but not in the corresponding lymph node metastasis (*KRAS*: $n = 10$; *BRAF*: $n = 1$; *PIK3CA*: $n = 6$), as far as available up to two additional lymph node metastases (*L2* and *L3*) were tested. All sequencing results are classified as either wild-type (*wt*) or mutant (*mut*). Samples representing the mutation status after first round of analysis (*C1*, *F1*, *L1*) are shaded in gray. Classifiers (*wt* or *mut*) of the second round of analysis and divergent from first run results are underlined.

and distant metastases were always identical. In all cases of discordant results, concerning the *KRAS* mutation status (wild-type versus mutated *KRAS*), either within primary tumors or when primary tumors were compared with lymph node or distant metastasis, discordancies did not result from incorrect standard cycle sequencing as confirmed by an independent pyrosequencing approach (Fig. 3).

BRAF. Of the 100 primary tumors, 7 (7%) exhibited *BRAF* mutations (Table 2), in line with the data reported in the literature (27–29). Heterogeneity between tumor centers and corresponding invasion fronts was observed in only one patient, in whom the mutation was restricted to the invasion front (Fig. 1B). Significant differences between the UICC tumor stages were not observed (Table 2). Comparing mutation patterns between primary tumors and corresponding lymph node metastases, in 2 of 55 cases (4% of matched pairs and 40% of matched pairs with mutated *BRAF*), *BRAF* mutations were exclusively restricted to the primary tumors (Fig. 1B). In only one of these cases, two additional lymph nodes could be analyzed in a second step, and both of them harbored mutated *BRAF* (Table 3). Heterogeneity between primary tumors and distant metastases was not observed (Fig. 1B). In all primary tumors and metastases, *BRAF* mutations were localized at codon 600.

PIK3CA. Under routine diagnostic tissue collection conditions (i.e., testing one single tumor sample of a given location), *PIK3CA* mutations were found in 21 (21%) of primary tumors (Table 2), consistent with previously reported results (30), and 5 of these (5% of all tumors and 24% of tumors with mutated *PIK3CA*) exhibited

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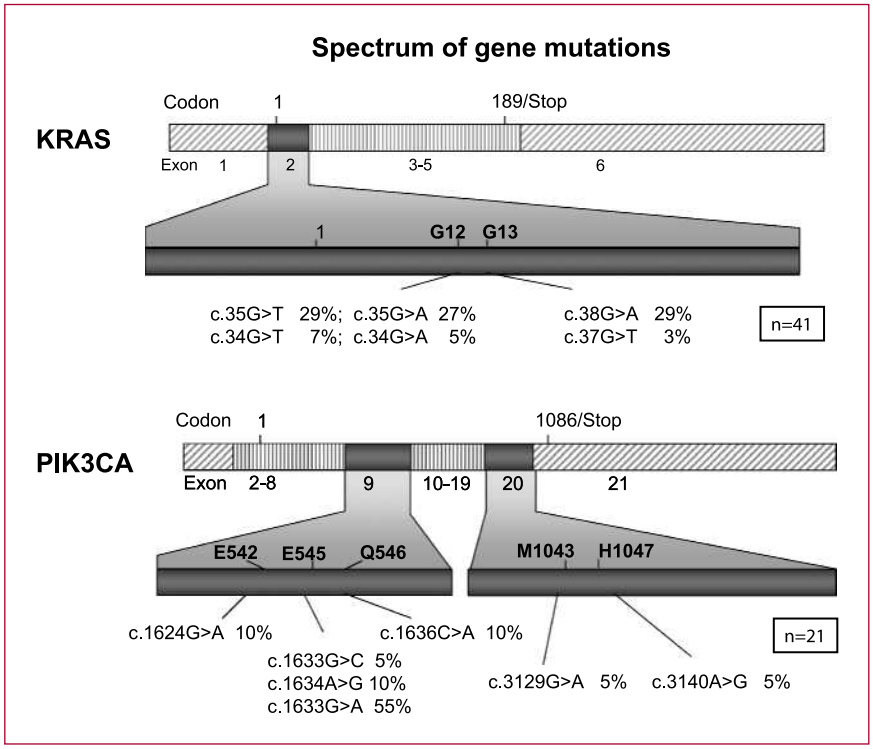

Fig. 2. Spectrum of KRAS and PIK3CA mutations in colorectal carcinomas. In 68% of the cases, *KRAS* mutations were localized at codon 12, whereas in 32% of the cases. *KRAS* mutations were localized at codon 13. Mutations in primary tumors and lymph node or distant metastases were always of the same type. In 90% of the cases, *PIK3CA* mutations were localized in exon 9, whereas in 10% of the cases, *PIK3CA* mutations were localized in exon 20. Mutations in primary tumors and lymph node or distant metastases were always of the same type.

heterogeneity between tumor centers and invasion fronts (Fig. 1C). Thus, in four patients (4% of all tumors and 19% the tumors carrying *PIK3CA* mutations), mutations were exclusively restricted to tumor centers, whereas in one patient (1% of all tumors and 5% of tumors with mutated *PIK3CA*), mutations were exclusively restricted to the invasion front. Significant differences between UICC tumor stages were not observed (Table 2).

Heterogeneity between primary tumors and lymph node metastases was detected in seven patients (13% of all 55 tumors for which matched pairs of primary tumors and lymph node metastases were available, and 50% of *PIK3CA*-mutated tumors of this subgroup). In six of these cases, the mutation was only found in the primary tumor, whereas in one case, it was restricted to the lymph node metastasis (Fig. 1C).

To investigate whether discordancies observed under the above-mentioned conditions were due to heterogeneity-related clonal selection, late-stage mutations during the process of metastasis, or sampling errors, additional tumor samples of these patients were analyzed. In the case of mutated *PIK3CA* in the lymph node metastasis, but wild-type *PIK3CA* in the primary tumor, two additional samples of the tumor center and invasion front each were tested. The *PIK3CA* mutation initially found in the lymph node metastasis could thereby also be identified in one of the four additionally tested primary tumor samples (Table 3). In all patients initially showing *PIK3CA* mutations in the primary tumors, but not in the lymph node metastases ($n = 6$), further lymph node metastases were analyzed. In three of these cases, *PIK3CA* mutations could be observed in at least one of two lymph node metastases (Table 3). In the other three cases, the additionally analyzed lymph node metastases exhibited *PIK3CA* wild-type, as was also found in the lymph node metastasis tested initially.

Comparing primary tumors and corresponding distant metastases ($n = 20$ cases) in terms of the *PIK3CA* mutation status, a discordant result with mutated *PIK3CA* in the primary tumor and wild-type *PIK3CA* in the liver metastasis was found in only one case (5% of all available matched pairs and 25% of all *PIK3CA*-mutated tumors; (Fig. 1C).

Considering all *PIK3CA* mutations identified in this study, these mutations affected different positions in exons 9 and 20, but 70% of all them were localized at codon 545 in exon 9 (Fig. 2). For a given patient, *PIK3CA* mutations in primary tumors, lymph node metastases, and distant metastases were always identical.

Concomitance of different mutations. Primary tumor specimens were also evaluated in terms of simultaneous occurrence of *KRAS, BRAF*, and/or *PIK3CA* mutations. Thus, no concomitant mutations of *KRAS* and *BRAF* genes were detected. In contrast, concomitant mutations of *PIK3CA* and *KRAS*, or *PIK3CA* and *BRAF* occurred in 9% and 3% of all cases, respectively. A mutation of at least one of the three genes was discovered in the majority (57%) of primary tumors, whereas 43% of primary tumors displayed no mutation in any of these genes. In stage III/stage IV tumors, the fraction of tumors showing at least one mutation of any of the three genes (66.7%) was significantly increased ($P = 0.017$, χ^2 test) when compared with stage I and II tumors (42.5%).

Clinicopathologic correlations. The mutation status of *KRAS, BRAF*, and *PIK3CA*, respectively, in primary tumors

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was correlated with the different clinicopathologic parameters (pT, pN, pM, and G). Thus, *KRAS* mutations correlated with a higher frequency of lymph node metastases (sum of pN1 and pN2 categories) compared with lymph node–negative (pN0) patients (50.0% versus 24.4%, $P = 0.014$). Considering the aspect of multiple testing, this result is of borderline significance. *BRAF* mutations were only observed in poorly differentiated (G3) carcinomas, but never in well- or moderately differentiated (G1 and G2) carcinomas (38.9% versus 0%, $P < 0.001$). Further correlations of *KRAS*, *BRAF*, or *PIK3CA* mutations with clinicopathologic data were not observed.

Discussion

KRAS mutations are regarded as an early event in the colorectal adenoma-carcinoma sequence, resulting in an important growth advantage in clonal expansions (39, 40) and can be found in ~40% of colorectal carcinomas. During the last years, targeted therapy with monoclonal antibodies (cetuximab and panitumumab) has been introduced into the therapy of metastatic colorectal cancer. Recent investigations revealed that colorectal cancer patients exhibiting *KRAS* mutations show no significant response to therapies with anti-EGFR antibodies. However, >50% of colorectal carcinomas exhibiting wild-type *KRAS* do not respond to this therapy either (5, 6, 14–20).

One possible reason for this unexpected therapeutic failure might be the absence of *KRAS* mutations in diagnostic samples due to tumor heterogeneity, as usually only one single tumor sample is analyzed under routine diagnostic conditions. In our series of 100 patients, tissue samples from both tumor centers and respective invasion fronts were analyzed separately. Thus, heterogeneity of *KRAS* mutations in primary tumors was found in 8% of all cases and 20% of tumors with mutated *KRAS*. Interestingly, a higher rate of *KRAS* mutations was detected in the tumor center compared with the invasion front, suggesting that diagnostic tumor samples should preferably be taken from the tumor center. Our results are in contrast to previous studies (31–33), which observed intratumoral heterogeneity of KRAS mutations in 35% to 47% of primary colorectal carcinomas. This, however, could be due to the low number of patients investigated in most of these studies (n = 19, 76, and 25, respectively). Interestingly, we observed a significantly higher proportion of *KRAS* mutations in primary tumors of stage III and IV compared with stage I and II patients. The same observation, supporting an evolutionary concept, was made in former studies, indicating that the rate of heterogeneity decreases during progression to higher stages of disease (33). Of note, except for one case, we did not observe heterogeneity in terms of *KRAS* mutations within stage IV primary tumors, suggesting that sampling errors are not likely to

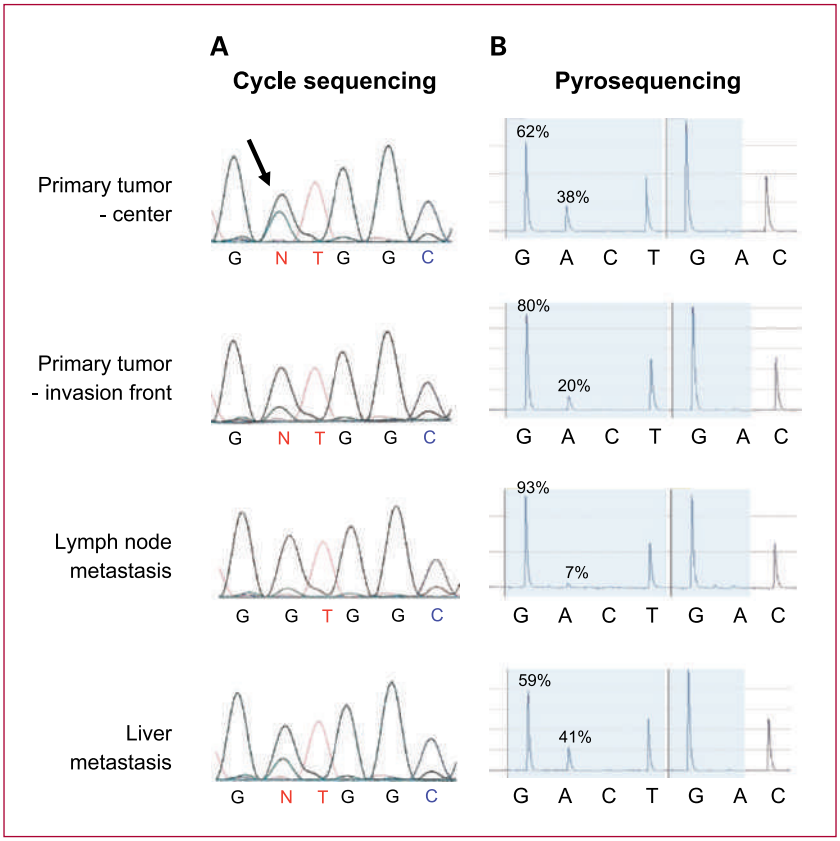

Fig. 3. KRAS mutation analysis by cycle sequencing and pyrosequencing. A, example of *KRAS* mutation analysis (patient no. 21) by standard cycle sequencing and pyrosequencing. Sequence data showed a mutation in codon 12 (c.35G>A) in the tumor center, at the invasion front, and in the liver metastasis, but the lymph node exhibited wild-type KRAS. Arrow, G to A transition at position 2 of codon 12. B, pyrosequencing of codon 12 and 13 confirmed the c.35G>A mutation, resulting in the exchange of glycine to aspartic acid at codon 12 in the respective tissue samples (percentage of mutant A-allele, >15%) and the wild-type status for the lymph node metastasis.

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explain the failure of EGFR antibody treatment in wild-type *KRAS* tumors of this stage.

Another clinically important aspect is heterogeneity of *KRAS* mutations between primary tumors, lymph node metastases, and distant metastases, as in clinical and diagnostic settings, usually only one single tumor sample is analyzed per patient, and thus far, no general recommendations exist as to which tumor samples (primary tumors, lymph node metastases, and distant metastases) should preferentially be tested. Therefore, in the present study, as far as available, not only primary tumors but also corresponding lymph node and distant metastases (one sample each) were analyzed. In a first step, matched pairs (n = 55) of primary tumors and lymph node metastases were compared. In 27% of these 55 cases and in 48% of tumors with mutated *KRAS*, respectively, *KRAS* mutations were detected only in the primary tumors, but not in the lymph node metastases, whereas 4% of the 55 cases and 6% of tumors with *KRAS* mutations showed an inverse pattern. These discordant results between primary tumors and lymph node metastases in terms of *KRAS* mutations did not result from incorrect standard cycle sequencing as verified by an independent pyrosequencing approach. Our results are in line with those of Oliveira et al. (41), who, by comparing 28 primary colorectal carcinomas and their corresponding lymph node metastases, observed a 32% rate of heterogeneity in terms of *KRAS* mutations. In contrast to our results, however, the overall rate of *KRAS* mutations detected in that study in primary tumors (64.3%) is much higher than the usually published rate of ~40%. This might be due to the small number of matched pairs of primary tumors and lymph node metastases (n = 28) included in that study.

To investigate whether the discordancies between primary tumors and lymph node metastases in terms of *KRAS* mutations were due to sampling errors, heterogeneity-related clonal selection, or late-stage mutations during the process of metastasis, additional tumor samples either from primary tumors or lymph node metastases of these patients were tested. In both cases initially classified as wild-type *KRAS* in the primary tumor, but mutated *KRAS* in the lymph node metastasis, subsequent analyses identified KRAS mutations in at least one of four additionally analyzed areas of primary tumors. This suggests that the lymph node metastases in these two cases resulted from minor *KRAS*-mutated subpopulations of the primary tumors during the process of metastasis rather than reflecting *de novo* mutations of *KRAS* within the lymph node metastases. Moreover, these results indicate that *KRAS* mutations, if present only in a minor subpopulation of the primary tumor, may remain undetected by the standard diagnostic procedures established thus far. If *KRAS* mutations were initially exclusively restricted to primary tumors, the analysis of additional lymph node metastases of the same patient finally led to the identification of the respective *KRAS* mutations in 6 of 10 cases. In the other four cases, wild-type *KRAS*, as found in the lymph node metastasis tested in the first place, was also identified in the additionally analyzed lymph node metastases. Our data indicate that different lymph node metastases of a given tumor with *KRAS* mutation may differ in their *KRAS* mutation status (possibly due to clonal selection during the process of metastasis), suggesting that lymph node metastases are not a reliable tool to determine the *KRAS* mutation status of colorectal cancer under routine diagnostic conditions.

In a next step, we also compared 20 primary tumors and corresponding distant metastases. We observed identical *KRAS* mutation status in 90% of the cases (18 of 20 patients). This is in accordance with recent publications reporting identical *KRAS* mutations in primary tumors and distant (mostly liver) metastases in approximately 90 to 100% of the cases (37, 42–44). Other authors reported a higher rate of heterogeneity between primary tumors and distant metastases (34). In summary, our data indicate that primary tumors as well as distant metastases seem to be the more reliable tissue specimens to define the *KRAS* mutation status in clinical practice than lymph node metastases.

As mentioned above, therapeutic failure of EGFR-specific antibody therapy in *KRAS* wild-type patients may result at least in part from activating mutations of *BRAF* or *PIK3CA*, respectively (22–24). However, studies on the heterogeneity of these mutations in colorectal cancer have neither been done for primary tumors nor compared with their corresponding metastases.

In our series, *BRAF* mutations were observed in 7% of the tumors, which is in line with a prevalence of 5 to 17% reported in the literature (27–29, 45–49). In our series, only one case (1% of all tumors and 14% of tumors with mutated *BRAF*) showed heterogeneity of *BRAF* mutations in primary colorectal carcinoma, which is far less than the rate of 40% observed by Giannini et al. (50) in multifocal papillary thyroid cancer. Comparing the mutation rates of *BRAF* in primary tumors and corresponding lymph node metastases, we found two patients (4% of all matched pairs and 40% of matched pairs with mutated *BRAF*) that harbored the mutation exclusively in the primary tumor, but not in the lymph node metastasis. In one of these patients, two additional lymph node metastases could be analyzed and both of them displayed mutated *BRAF*. A heterogeneous distribution of *BRAF* mutations between primary tumors and distant metastases was not observed in our series, which is in accordance with the data of Molinari et al. (44). In summary, *BRAF* mutations are rare in colorectal carcinomas, but if present, a significant proportion (14%) of *BRAF*-mutated tumors will erroneously be misdiagnosed as wild-type due to intratumoral heterogeneity and consecutive sampling errors. Similarly, as outlined for the *KRAS* mutation status, in a clinical and diagnostic setting, the *BRAF* mutation status should not be determined from lymph node metastases, as clonal selection contributes to a high rate of heterogeneity.

Concerning *PIK3CA*, we found mutations in 21% of primary colorectal carcinomas, which is in accordance with other reports (30, 45, 51–54). Thus far, no data on heterogeneity of the *PIK3CA* mutation status have been published. In our series, 5% of the patients showed heterogeneity of

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PIK3CA mutations as primary tumors were concerned. Heterogeneity between primary tumors and the corresponding lymph node metastases was observed in 13% of all matched pairs available ($n = 55$), which account for 50% of *PIK3CA*-mutated tumors of this subgroup. As for *KRAS*, in the majority of these cases, the mutations were found in the primary tumor but not in the corresponding lymph node metastases. However, as already shown for *KRAS* and *BRAF*, the analysis of additional lymph node metastases lead to the detection of a higher rate of *PIK3CA* mutations. There was no significant heterogeneity between primary tumor and distant metastasis with only 1 case of 20 showing a *PIK3CA* mutation in the primary tumor but not in the distant metastasis. Therefore, in clinical practice, *PIK3CA* mutation testing of primary tumors as well as distant metastases are appropriate tools to define the *PIK3CA* mutation status before EGFR-targeted therapy, whereas the analysis of a single lymph node metastases is less representative for the entire tumor and, therefore, should not be done in the first place.

In summary, we observed a high rate of *KRAS* mutations (41%), a moderate rate of *PIK3CA* mutations (21%), and a low rate of *BRAF* mutations (7%) in a cohort of 100 primary colorectal carcinomas. Because some mutations occurred concomitantly in a given patient, which is in line with recent publications (28, 51), in total, 57% of all patients showed at least one mutation of any of these three genes by testing two different areas of primary tumors. This rate could slightly be increased to 60% by analyzing additional primary tumor samples. Therefore, our data suggest that including mutation analyses for *BRAF* and *PIK3CA* in addition to *KRAS* into a standard diagnostic setting of colorectal cancer would allow the identification of about another 15% of patients who will probably not benefit from EGFR-specific antibody therapy. However, this has to be verified in prospective clinical studies. Considering the high costs of such a therapy and the comparably low costs of these molecular tests, it might be economically reasonable to include these analyses into a standard diagnostic setting of *KRAS* wild-type tumors. The mutation rates of *BRAF* and *PIK3CA*, however, do not sufficiently explain the high rate of 50% treatment failure in *KRAS* wild-type tumors, suggesting that additional members of EGFR-dependent signaling pathways might be mutated or somehow constantly altered, depending on their activating or inhibiting function. In this context, mutation or lack of expression of *PTEN*, overexpression of pAkt, increased *EGFR* or *HER2* gene copy number, increased EGFR phosphorylation, and overexpression of alternative EGFR ligands such as amphiregulin or epiregulin are discussed in the literature (55). Moreover, we could show that in tumors harboring mutations of any of the three genes studied, significant rates of heterogeneity (up to 50%) occur. These are most pronounced when primary tumors and lymph node metastases were compared and most likely result from clonal selections during the process of metastasis. As a consequence, in a clinical and diagnostic setting, mutational analyses should preferentially be done on samples of primary tumors or distant metastases, whereas lymph node metastases seem to be a less appropriate tool.

Disclosure of Potential Conflicts of Interest

K.-L. Schaefer and H.E. Gabbert are members of the Amgen advisory board.

Acknowledgments

We thank Ingrid Büchmann, Julia Lange, and Marianne Niermann-Kaiser for their excellent technical assistance.

Grant Support

Forschungskommission of the Medical Faculty of the University of Duesseldorf (S.E. Baldus and K.-L. Schaefer) and VFK Krebsforschung, Berlin (N.H. Stoecklein).

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Received 9/9/09; revised 11/11/09; accepted 11/13/09; published OnlineFirst 1/26/10.

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Prevalence and Heterogeneity of *KRAS, BRAF*, and *PIK3CA* Mutations in Primary Colorectal Adenocarcinomas and Their Corresponding Metastases

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Clin Cancer Res 2010;16:790-799. Published OnlineFirst January 26, 2010.

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