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ORIGINAL RESEARCH

Comparison of *KRAS* and *PIK3CA* gene status between primary tumors and paired metastases in colorectal cancer

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Purpose: In metastatic or recurrent colorectal cancer (MRCRC), the concordance of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutation status between the primary tumors and metastases is still controversial. The purpose of this study was to evaluate the association between *KRAS* and *PIK3CA* mutational status and various clinicopathologic features, and compare their genotype in primary tumors with that of the paired metastatic tumors.

Method: We compared the mutation status of *KRAS* and *PIK3CA* between the primary tumors and the paired metastases of 59 MRCRC patients with available tissues (resection or biopsy). The presence of *KRAS* and *PIK3CA* mutations were determined by direct sequencing analysis.

Results: Seventeen patients (28.8%) had the *KRAS* mutation and 46 patients (80.0%) had the *PIK3CA* mutation when considering both the primary and metastatic sites. *KRAS* mutation was observed in ten primary tumors and eleven related metastases (16.9% vs 18.6%), while *PIK3CA* mutation was found in 26 primary tumors and 32 related metastases (44.1% vs 54.2%). *KRAS* status was concordant between primary and metastatic sites in 45 patients (76.3%, kappa =0.157), while the concordance of *PIK3CA* status was only found in 25 patients (42.4%, kappa =–0.141). The *PIK3CA* status discordance rate was significantly higher in 40 patients undergoing metachronous resection of primary tumor or metastasis, compared with that in 19 patients with synchronous resection of primary tumor or metastasis (67.5% [27/40] vs 36.8% [7/19]; *P*=0.026).

Conclusion: Our results demonstrate that low concordance of *KRAS* and high discordance of *PIK3CA* mutational status exist between the primary tumors and paired metastasis, and these findings remind us to have second thoughts about the need to evaluate metastatic tumors separately rather than only based on the primary tumor data when targeted therapy is considered.

Keywords: *KRAS*, *PIK3CA*, colorectal cancer, primary tumor, metastatic site

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related deaths worldwide. Despite improvements made in therapy refinement, 25% of patients are diagnosed at IV stage of the disease, and approximately 50% of patients develop into metastatic or recurrent colorectal cancer (MRCRC) even though they received curative resection of primary CRC during the first 5 years from diagnosis.[1] For MRCRC patients, systematic therapy containing chemotherapy, radiotherapy, and targeted therapy was considered to be the major treatment. As for approximately 60%–70% Kirsten rat sarcoma viral oncogene homolog (*KRAS*) wild-type MRCRC, anti-epidermal growth factor receptor (anti-EGFR) inhibitors, such as cetuximab and

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panitumumab might enhance antitumor effects combined with chemotherapy according to recent guidelines.[2] However, the response rate is not high, nearly 50% *KRAS* wild-type MRCRC patients cannot benefit from these combined therapies with anti-EGFR inhibitors.[3–5] Emerging data have proposed phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutation might account for nonresponders to EGFR targeting in CRC.[6–8] For example, De Roock et al[9] found that patients with *PIK3CA* mutations had a significantly lower response rate in *KRAS* wild-type patient.

Furthermore, in clinical practice, most results of gene tests were carried out mainly on primary tumors due to the difficulties to obtain tissues of metastatic tumors. However, some research results raise concerns that genetic profiling of primary tumors may not be representative of metastatic disease.[10,11] In MRCRC, the concordance of gene mutation status between the primary tumors and metastases is controversial. Jones et al[12] found a high degree of concordance between primary tumors and metastases. In contrast, Vermaat et al[13] reported a high degree of mutational discordance between primary and metastatic samples using next-generation sequencing. Some study reports have shown gene mutations, such as *KRAS*, *NRAS*, and *BRAF* were highly concordant between primary tumors and metastases,[14,15] while discordant mutations were observed in genes of the phosphoinositide 3-kinase pathway.

Consideration of the above phenomenon, the heterogeneity between primary tumors and metastases seemed as an additional reason for the failure of targeted therapies in MRCRCs. Thus, our study was aimed to evaluate the genetic relationship between primary MRCRCs and their matched metastases that will consequently help in targeted therapy.

Materials and methods

Study population

Among patients with histologically confirmed colorectal adenocarcinoma who had been treated or followed up at Zhejiang Cancer Hospital between June 2004 and July 2013, 59 MRCRC patients who had undergone surgical resection or biopsy of both primary tumors and related metastatic sites were enrolled. The retrospective study was performed using the stored samples at the Department of Pathology, and all of the samples were from patients who had received surgical resection or biopsy of both primary and related metastatic tumors with their consent, and anonymized before the study. The patient did not provide written informed consent in our study. The study was approved by the medical ethics committee at Zhejiang Cancer Hospital.

Table 1 The PCR primers for *KRAS* and *PIK3CA* gene amplification

Gene	Primers
KRAS	
Codons	5′-AGGTACTGGTGGAGTATTTGATAGTGT-3′ (forward)
12, 13	5′-CCTCTATTGTTGGATCATATTCGTC-3′ (reverse)
Codons 61	5′-GGTGCACTGTAATAATCCAGACT-3′ (forward)
	5′-CATGGCATTAGCAAAGACTCA-3′ (reverse)
Codons	5′-AGACACAAAACAGGCTCAGGA-3′ (forward)
117	5′-TTGAGAGAAAAACTGATATATTAAATGAC-3′ (reverse)
PIK3CA	
Codons	5′-AGACACAAAACAGGCTCAGGA-3′ (forward)
545	5′-TTGAGAGAAAAACTGATATATTAAATGAC-3′ (reverse)

Abbreviations: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; PCR, polymerase chain reaction; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

Sequence analysis of *KRAS* and *PIK3CA* gene

Formalin-fixed paraffin-embedded primary tumor and metastatic tissue specimens were microdissected manually under the supervision of experienced gastrointestinal pathologists. The DNA was extracted according to the manufacturer's instructions of E.Z.N.A.FFPE DNA Kit (Lot. D3399-01, OMEGA Bio-Tek, Norcross, GA, USA). For mutation analyses, extracted tumor DNA samples were amplified by polymerase chain reaction (PCR) using primers (Table 1). The PCR conditions were as follows: one cycle of 95°C for 5 minutes; 34 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and one cycle of 72°C for 10 minutes. The PCR products were determined by 3% agarose gel electrophoresis and then sequenced using the same forward primer of each gene by Invitrogen 3730XL genetic analyzer (Life Technologies, Carlsbad, CA, USA). The sequencing results were analyzed with Chromas software under the condition of signal-to-noise ratio >98%.

Statistical analysis

The concordant rate of *KRAS* and *PIK3CA* mutational status in primary tumors and related metastases was evaluated, the Kappa index was measured using Cohen's kappa coefficient, which can assess the concordance between categorical variables of the same individuals.[16] The effect of *KRAS* and *PIK3CA* mutational status on clinicopathologic features was assessed using Pearson's chi-square or Fisher's exact tests. The relationship between the discordant rates of the *KRAS* and *PIK3CA* mutation status and various clinicopathologic

features was also evaluated using univariate analyses (Pearson's chi-square or Fisher's exact tests). $P<0.05$ was considered significant. All analyses were performed using SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics (N=59) and the association between *KRAS* or *PIK3CA* mutational status and clinicopathologic features

Of the 59 MRCRC in this study, 30 patients (50.8%) were male. The median age was 46 years (range, 18–80 years). Related metastatic sites for gene analysis were liver (N=33), lung (N=9), peritoneum (N=4), pelvic (N=4), ovary/uterus (N=6), groin (N=1), stomas (N=1), and pancreas (N=1), respectively. The primary tumor sites were right colon in 13 patients (22.0%), left colon in 15 patients (25.4%), rectum in 29 patients (49.2%), and others in two patients (3.4%). Other patient characteristics are shown in Table 2. Age in MRCRC patients was different according to the *KRAS* mutational status. Age <60 was more frequent in wild-type *KRAS* patients than in mutant *KRAS* patients (83.3% vs 16.7%; $P=0.010$). There was no association between the *KRAS* or *PIK3CA* status and other clinicopathologic features (sex, primary tumor location, histology and grade, clinical situations for the development of systemic metastasis, and metastasis sites) (Table 2).

Frequency and types of *KRAS* and *PIK3CA* mutation

Ten (16.9%) patients with *KRAS* mutation and 26 (44.1%) patients with *PIK3CA* mutation were observed in primary tumors, with eleven (18.6%) and 32 (54.2%) in related metastatic sites, respectively. Seventeen patients (28.8%) had the *KRAS* mutation and 46 patients (80.0%) had *PIK3CA* mutation in any place of the primary or metastatic sites. Of those 17 patients with *KRAS* mutation, two patients had a *KRAS*12 codon GGT-AGT mutation, five had a *KRAS*12 codon GGT-GCT mutation, four had a *KRAS*12 codon GGT-GAT mutation, three had a *KRAS*12 codon GGT-GTT mutation, two had a *KRAS*13 codon GGC-GAC mutation (one combined with *KRAS*12 mutation), one had a *KRAS*61 codon CAA-CAT mutation, and one patient had a *KRAS*117 codon AAA-ATA mutation. In addition, of those 46 patients with *PIK3CA* mutation, 45 patients had a *PIK3CA*545 codon

Table 2 Patient characteristics and the association between *KRAS* or *PIK3CA* status and clinicopathologic parameters

Characteristic	Number of patients						
	All	*KRAS*	*KRAS*	*P*-value	*PIK3CA*	*PIK3CA*	*P*-value
	N	WT	MT		WT	MT	
		N (%)	N (%)		N (%)	N (%)	
Sex				0.054			0.701
Male	30	18 (60.0)	12 (40.0)		6 (20.0)	24 (80.0)	
Female	29	24 (82.8)	5 (17.2)		7 (24.1)	22 (75.9)	
Age				0.010			0.548
<60 years	36	30 (83.3)	6 (16.7)		7 (19.4)	29 (80.6)	
≥60 years	23	12 (52.2)	11 (47.8)		6 (26.1)	17 (73.9)	
Primary tumor location				0.777			0.563
Right	13	9 (69.2)	4 (30.8)		2 (15.4)	11 (84.6)	
Left	15	12 (80.0)	3 (20.0)		5 (33.3)	10 (66.7)	
Rectum	29	20 (70.0)	9 (30.0)		6 (20.7)	23 (79.3)	
Others	2	1 (50.0)	1 (50.0)		0 (0)	2 (100)	
Metastasis				0.088			0.410
Synchronous	24	20 (83.3)	4 (16.7)		4 (16.7)	20 (83.3)	
Metachronous	35	22 (62.9)	13 (37.1)		9 (25.7)	26 (74.3)	
Histology and grade				0.885			0.787
Well/moderately differentiated	39	28 (71.8)	11 (28.2)		9 (23.1)	30 (76.9)	
Poorly differentiated, mucinous	20	14 (70.0)	6 (30.0)		4 (20.0)	16 (80.0)	
Resection style				0.770			0.584
Concurrent	19	14 (73.7)	5 (26.3)		5 (26.3)	14 (73.7)	
Subsequent	40	28 (70.0)	12 (30.0)		8 (20.0)	32 (80.0)	

Abbreviations: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; WT, wild-type; MT, mutation type.

Table 3 Distribution of *KRAS* mutation types

Gene	Mutation types	Patients (N)	%
KRAS (28.8%)	*KRAS* 12 codon GGT-ACT	2	11.76
	KRAS 12 codon GGT-GCT	5	29.41
	KRAS 12 codon GGT-GAT	4	25.53
	KRAS 12 codon GGT-GTT	3	17.65
	KRAS 13 codon GGC-GAC	2*	5.88
	KRAS 61 codon CAA-CAT	1	5.88
	KRAS 117 codon AAA-ATA	1	5.88
PIK3CA (80.0%)	*PIK3CA* 545 codon CAG-GCG	45	97.83
	PIK3CA 545 codon CAG-AAG	1	2.17

Note: *One patient combined with *KRAS* 12 mutation.
Abbreviations: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

CAG-GCG mutation and one had a *PIK3CA* 545 codon CAG-AAG mutation (Table 3).

Concordance of *KRAS* and *PIK3CA* status in primary tumors and related metastases

KRAS status was concordant between primary and metastatic sites in 45 patients (76.3%; kappa =0.157). Of the 14 discordant cases, seven patients had the *KRAS* mutation in the primary tumors, but not in the metastatic sites; seven patients had the *KRAS* mutation only in the metastatic tumors and not in the primary tumors. *PIK3CA* status was low concordant between primary and metastatic sites in 25 patients (42.4%; kappa =–0.141). Of the 34 discordant cases, 14 patients had the *KRAS* mutation in the primary tumors, and not in the metastatic sites; 20 patients had the *KRAS* mutation in the metastatic tumors without in the primary tumors (Table 4).

Discordance rates of *KRAS* and *PIK3CA* status according to various clinicopathologic features

We evaluated the discordance rates of *KRAS* and *PIK3CA* mutation status between primary tumors and paired tissues with various clinicopathologic factors such as primary tumor location, metastatic organs, type of primary or metastatic tumor specimens (biopsied vs resected), and any chemotherapy/radiotherapy between resection of primary and metastasis before obtaining primary or metastatic tumor specimens or resection style. The lung was the most frequent site showing *KRAS* discordance, however, no difference was seen in the discordance rate of *KRAS* status for the metastatic organs. Two patients with tissues acquired from primary tumor (left colon and rectal colon) and related stomas and groin metastatic site showed discordance of *KRAS* status (mutation type [P]-wild-type [M]). One patient with tissues acquired from primary tumor (transverse colon) and related pancreatic metastatic site showed concordance of *PIK3CA* status (mutation type [P]-mutation type [M]). Otherwise, the discordant rate of *PIK3CA* status was frequent (liver, 60.6%; lung, 55.5%; and peritoneum, 50.0%) regardless of related metastatic site. Two patients with tissues acquired from primary tumor (transverse colon and left colon) and related pancreatic and stomas metastatic site showed discordance of *PIK3CA* status (mutation type [P]-wild-type [M]). One patient with tissues acquired from primary tumor (rectal colon) and related groin metastatic site showed concordance of *PIK3CA* status (mutation type [P]-wild-type [P]). Additional analyses were performed to find whether the discordance rates of *KRAS* and *PIK3CA* status were influenced by other various clinicopathologic factors (67.5% vs 36.8%; *P*=0.026). However, there was no difference in the discordant rate of *KRAS* and *PIK3CA* status for other clinicopathologic factors (Table 5).

Table 4 *KRAS* and *PIK3CA* mutational status of primary tumors and paired metastatic sites

Primary sites	Metastatic sites	
	No of wild-type	No of mutation type
KRAS		
No of wild-type	42	7
No of mutation type	7	3
PIK3CA		
No of wild-type	13	20
No of mutation type	14	12

Abbreviations: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; No, number.

Discussion

EGFR monoclonal antibodies, such as cetuximab and panitumumab are currently approved for the treatment of metastatic CRC patients with *KRAS* wild-type tumors. However, their antitumor activity has been limited by intrinsic and acquired drug resistance. One explanation for drug resistance is cancer genetic heterogeneity, which contains content of two aspects.[13,17] One refers CRC patients can harbor different gene mutations between primary tumors and paired metastatic sites, another means that even gene disaccordance existed within different regions of the same tissues. Heterogeneous in genes have been reported to play a role in resistance to anti-EGFR drugs in CRC, including activating mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*.[9] Genetic heterogeneity makes it more difficult to decide to use the anti-EGFR drugs only based on the results of gene test either from primary or metastatic tumor. Therefore, we conducted the study to compare these gene statuses between

Table 5 Univariate analyses on the association between clinicopathologic features and the discordance rates of *KRAS* and *PIK3CA* mutation status

Gene	Characteristic	Univariate analysis		
		Concordant cases, N (%)	Discordant cases, N (%)	*P*-value
	Metastatic site			0.271
	Liver	25 (75.8)	8 (24.2)	
	Lung	6 (66.7)	3 (33.3)	
	Peritoneum/ovary/uterus/pelvic	13 (92.9)	1 (7.1)	
	Others	1 (33.3)	2 (66.7)	
	Primary tumor location			0.677
	Right	9 (90)	1 (10)	
	Left	11 (78.6)	3 (21.4)	
	Rectum	21 (72.4)	8 (27.6)	
KRAS	Type of tumor specimens			0.095
	Resected	32 (71.1)	13 (28.9)	
	Biopsied	13 (92.8)	1 (7.1)	
	Any chemotherapy/radiotherapy between resection of primary and metastasis			0.333
	Yes	29 (80.6)	7 (19.4)	
	No	16 (69.6)	7 (30.4)	
	Resection style			0.748
	Synchronous	14 (73.7)	5 (26.3)	
	Metachronous	31 (77.5)	9 (22.5)	
	Metastatic site			0.794
	Liver	13 (39.4)	20 (60.6)	
	Lung	4 (44.4)	5 (55.6)	
	Peritoneum/ovary/uterus/pelvic	7 (50.0)	7 (50.0)	
	Others	1 (47.1)	2 (52.9)	
	Primary tumor location			0.606
	Right	3 (30)	7 (70)	
	Left	7 (50)	7 (50)	
	Rectum	13 (44.8)	16 (55.2)	
PIK3CA	Type of tumor specimens			0.564
	Resected	20 (45.4)	25 (55.6)	
	Biopsied	5 (35.7)	9 (64.3)	
	Any chemotherapy/radiotherapy between resection of primary and metastasis			0.223
	Yes	13 (36.1)	23 (63.9)	
	No	12 (52.2)	11 (47.8)	
	Resection style			0.026
	Synchronous	12 (63.2)	7 (36.8)	
	Metachronous	13 (32.5)	27 (67.5)	

Abbreviations: *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

primary tumors and paired metastasis in CRC for developing effective therapeutic strategies. We evaluated all the genes statuses, such as *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* in primary CRC and their matched metastasis, and compared the discordance between the two sites. However, only one patient with *NRAS* mutation was found in metastatic site and no *BRAF* mutation was detected due to the relatively small sample size. Subsequently, we performed direct sequencing of *KRAS* and *PIK3CA* genes in 59 primary CRC tumors and matched metastases to define the mutational concordance of these genes in primary and metastatic tumors. The *KRAS* or *PIK3CA* mutational statuses were regarded as positive if they were mutated in any place of primary tumors or related metastases in our study. Approximately 28.8% of CRCs harbor *KRAS* mutations, which is almost consistent with prior studies. While *PIK3CA* mutations were detected in 80% of patients with 44.1% of primary tumors and 54.2% of related metastatic sites, respectively, which showed relatively higher mutational rate than that of prior reports.[18,19] Approximately more than 80% *KRAS* mutations were detected in codons 12, 13 in exon 2, and our results showed 100% mutation analyses in codons 545 of the *PIK3CA* gene

in exon 9. Furthermore, ten patients out of 59 in our study harbored both *KRAS* and *PIK3CA* mutations, which may be a certain cluster having the low response to EGFR-targeted treatment and a poor prognosis, which can be deduced from the previous reports.

Our findings demonstrate a high level of concordance of *KRAS* mutation status and a significant discordance of *PIK3CA* mutation status in primary tumors and matched metastases, which is in agreement with previous studies.[20,21] *KRAS* status was found to be concordant in 76.3% of the analyzed primary and metastatic sites in 59 patients, while *PIK3CA* gene discordant rate was up to 57.6% in our study. As the sample size was small (N=59), we did not find difference of gene mutational rate of *KRAS* and *PIK3CA* in the primary tumors and metastasis in these 59 patients. Nevertheless, *PIK3CA* gene mutational rate was more frequently observed in metastatic specimens than primary sites (61.5% vs 44.6%; *P*=0.025) in our unselected CRC patients (table not shown). De Roock et al[9] reported that *PIK3CA* mutations may negatively impact the response to EGFR inhibitors of CRC patients, and *PIK3CA* exon 20 mutations were also significantly associated with shorter survival.[7] Also, Domingo et al[22] found that patients with *PIK3CA* mutant could benefit from aspirin therapy after CRC diagnosis and had a reduced rate of CRC recurrence. Therefore, our findings indicated that if patients with wild-type *KRAS* were selected to receive EGFR-targeted therapy, it could be more appropriate to perform *KRAS* and *PIK3CA* genotyping in both the primary tumors and metastases. Baldus et al[21] previously reported the obvious discordance of the status of gene involved in *PIK3CA*/AKT pathway between primary tumors and metastasis, also with relative higher mutational rate in metastatic sites. Moreover, acquisition of new mutations may be developed during the evolution of the metastatic process.[23] It is obvious that it remains to be proven in prospective well-designed clinical studies.

We also discussed the main influencing factors resulting in genetic heterogeneity between primary and metastatic tissues. As previously reported, discrepancy may be related to the different sites of primary tumor location, the metastatic organs, type of tumor specimens (resected or biopsied), any therapy between resection of primary and metastasis or resection style (synchronous or metachronous) (Goswami et al,[23] 2014 ASCO Annual Meeting). However, in our study, we found no difference in the discordant rate of *KRAS* and *PIK3CA* status with these clinicopathologic factors except the paired specimens obtained from metachronous resection showed an increased *PIK3CA* discordant rate compared with synchronous resection. Goswami et al reported that metachronous resection of the two sites, receiving intervening chemotherapy between resection of primary and metastasis and even increasing number of lines of intervening chemotherapy, can be the possibility for the observed discordance in the *KRAS* and *PIK3CA* mutation status.[23] The rates of *KRAS* and *PIK3CA* gene discordance between primary and metastasis vary by the related metastatic sites, with a relatively higher rate of discordance in brain, bone, peritoneum, or lung metastases when compared with other metastatic organs including liver, distant LN, or ovary.[24] Therefore, the causes of discordance need to be further evaluated in future larger studies.

Our study shares several limitations common to the majority of published findings in this field. First, our mutational analysis was performed by the traditional sequencing analysis with relatively low sensitivity, and not confirmed by other more sensitive methods as amplification-refractory mutation system allele-specific PCR combined with Scorpions probes or peptide nucleic acid-clamp allele-specific reverse transcription-PCR assay. Second, the retrospective analysis is prone to bias or error, and the sample is relatively small. Third, we did not conduct the repetitive and multipoint mutational analysis of the same tissue, which may lead to the false-negative results. Despite these limitations, our study provides some clinically meaningful suggestions. This study demonstrated the existence of a significant discordance of *PIK3CA* and relative concordance of *KRAS* mutations occurring in primary tumors and their corresponding metastases in patients with CRC. These reminded that gene test can not only be conducted in primary tissues, but the metastatic specimen also needs to be reexamined if the tissue is available. Our study also raised the hypothesis that combined analysis of *KRAS* and *PIK3CA* to select the proper CRC patients will be an effective strategy for EGFR-targeted therapy, which remains to be proven in well-designed clinical studies.

In conclusion, our findings indicate a concordance of *KRAS* mutation and a discordance of *PIK3CA* mutation between the primary tumors and the matched metastases in CRC and suggest that status of specific molecules in metastatic tumors need to be reevaluated when the patients with metastases are about to use the EGFR-targeted therapy.

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Author contributions

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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