

# Analysis of KRAS, BRAF, PTEN, IGF1R, EGFR intron 1 CA status in both primary tumors and paired metastases in determining benefit from cetuximab therapy in colon cancer

Jin Hyun Park · Sae-Won Han · Do-Youn Oh ·  
Seock-Ah Im · Seung-Yong Jeong · Kyu Joo Park ·  
Tae-You Kim · Yung-Jue Bang · Jae-Gahb Park

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## Abstract

**Purpose** The aim of this study was to determine the expression of molecular markers in metastatic colorectal cancer (mCRC) and the concordance between primary tumor and metastasis. We also aimed to determine the relationship between molecular markers and clinical outcomes of cetuximab-containing chemotherapy.

**Methods** Seventy-five mCRC patients who received cetuximab-containing chemotherapy between 2000 and 2008 were consecutively enrolled. EGFR, p-EGFR, PTEN, and IGF-1R expression by immunohistochemistry, DNA sequencing for EGFR, KRAS, BRAF, and PI3 KCA, and EGFR amplification by FISH were done.

**Results** The positive expression of EGFR, p-EGFR, PTEN, and IGF-1R was determined in 45 (64.3%), 9 (14.8%), 35 (50.7%), and 10 patients (16.1%), respectively. EGFR gene amplification or high polysomy was detected in 10 patients (17.6%). KRAS mutation and BRAF mutation were detected in 19 patients (27.5%) and five patients (7.0%), respectively. Among tested biomarkers, only the EGFR intron 1 CA repeat polymorphism and BRAF mutation showed concordance ( $\kappa = 0.600$ ,  $P = 0.003$ ;

and  $\kappa = 0.692$ ,  $P = 0.001$ , respectively) between primary tumor and paired metastasis. Skin rash was a strong predictive marker for response rate, PFS, and OS. In KRAS mutant tumors, PTEN expression was associated with a longer PFS. BRAF mutation was related to poor outcome in KRAS wild-type tumors.

**Conclusions** BRAF mutations and EGFR intron 1 CA repeat polymorphisms were concordant between primary tumors and paired metastases. In KRAS mutant tumors, PTEN expression was a predictive marker for favorable outcomes. In KRAS wild type, BRAF mutation was strong predictive markers for poor outcomes.

**Keywords** Colon cancer · Cetuximab · KRAS · BRAF · PTEN · EGFR intron 1 CA

## Introduction

Since cetuximab was introduced for the treatment of mCRC, there have been many studies aimed at identifying the predictive markers for chemotherapeutic responsiveness. Several studies have shown, by immunohistochemistry (IHC), that EGFR protein expression in cancer tissues does not predict the response to cetuximab treatment [1]. In contrast, a gain in the EGFR gene copy number due to polysomy or gene amplification, which has been evaluated by fluorescent in situ hybridization (FISH), seems to be a better predictive marker for response to anti-EGFR therapy [2, 3]. KRAS mutation and PTEN loss by IHC are well known as predictive markers for poor outcome to cetuximab treatment [4]. BRAF and PIK3CA mutations are considered to predict a poor response to cetuximab treatment [5, 6].

IGF1R results in upregulation in the majority of CRCs, most likely contributing to the aggressive growth

J. H. Park · S.-W. Han · D.-Y. Oh (✉) · S.-A. Im ·  
T.-Y. Kim · Y.-J. Bang  
Division of Medical Oncology, Department of Internal  
Medicine, Seoul National University Hospital, 101 Daehang-ro,  
Jongno-gu, Seoul 110-744, Republic of Korea  
e-mail: ohdoyoun@snu.ac.kr

S.-W. Han · D.-Y. Oh · S.-A. Im · T.-Y. Kim · Y.-J. Bang  
Cancer Research Institute, Seoul National University  
College of Medicine, Seoul, Republic of Korea

S.-Y. Jeong · K. J. Park · J.-G. Park  
Department of Surgery, Seoul National University Hospital,  
Seoul, Republic of Korea

characteristics of the tumor and poor prognosis [7, 8]. Retrospective data suggest that overexpression of IGF1R is a potential predictive factor for cetuximab treatment [9].

The number of CA single sequence repeats in intron 1 of the EGFR gene, which affects the transcription efficiency of the gene, is associated with the response to EGFR antibodies. Several data suggest a correlation between polymorphic variations in intron 1 of the EGFR gene and the response to EGFR inhibitors [10].

Most of these data were obtained by analyzing the clinical outcomes and molecular features in primary tumors. It is possible, however, that primary tumors and paired metastatic lesions might be different with respect to molecular marker expression or gene status and that these differences may affect the clinical significance of a predictive test. Few data exist regarding the concordance of expression of molecular markers between primary sites and paired metastases. Furthermore, little is known about predictive molecular markers for cetuximab treatment in Eastern countries.

We conducted this study to determine the variable expression of molecular markers that could affect the response to cetuximab treatment in Eastern countries and to determine whether or not the status of molecular markers between primary tumors and paired metastases is concordant. We also determined the predictive markers for cetuximab treatment.

## Patients and methods

We consecutively enrolled CRC patients who underwent cetuximab-containing palliative chemotherapy at Seoul National University Hospital between 2000 and 2008, and from whom tumor tissue specimens were available.

All patients were treated with cetuximab with or without combined cytotoxic chemotherapy. The initial cetuximab dose was 400 mg/m<sup>2</sup>, followed by 250 mg/m<sup>2</sup> every week. Treatment was continued until disease progression (PD), intolerable toxicity, or patient refusal.

### Clinical evaluation and response criteria

The response was evaluated every 6 weeks with computed tomography according to response evaluation criteria in solid tumors (RECIST). The patients who had a complete response (CR) or partial response (PR) were defined as responders. The disease control rate reflects the proportion of the patients who had a CR, PR, and stable disease (SD).

### Molecular analysis

Paraffin-embedded tumor specimens were used to construct a tissue microarray with 2-mm-diameter cores in tissues

which were operative specimens. In biopsy specimens, all tissues were used. Genomic DNA was extracted using the QIAamp Mini Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions. For patients with tissues from primary tumors and corresponding metastases, the sample from the primary lesion was used to represent the patient in the correlative analyses with a clinical outcome.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed on a Leica Bond-max automated immunostainer (Leica Microsystems, Newcastle, UK) following the manufacturer's protocols.

The following antibodies were used: EGFR (clone 31G7; Zymed, San Francisco, CA, USA), p-EGFR (clone 1H12; Cell Signaling, Beverly, MA, USA), PTEN (clone 6H2.1; Dako, Glostrup, Denmark), and IGF1R ( $\beta$  subunit; Cell Signaling). Antibody binding was detected using the Bond Polymer Refine Detection kit (Leica Microsystems).

### Interpretation of IHC

EGFR was assessed according to the EGFR pharmDx<sup>TM</sup> scoring guidelines. PTEN staining was mainly cytoplasmic. The intensity was scored according to a four-tier system, as follows: 0, no staining; 1, weak; 2, moderate; and 3, strong. We assigned 1, 2, or 3 additional points if the percentage of positive cells was <25, 25–50, or >50%, respectively. Specimens were defined as positive if the score was  $\geq 4$  [11].

To detect tyrosine phosphorylation of EGFR, only membranous staining was considered positive for EGFR. A cut-off value of 10% positive cells with an intensity  $>2$  was defined as a positive expression of EGFR [12].

The staining of IGF1R protein (membranous and/or cytoplasmic) was also semi-quantitatively defined as a positive expression based on  $>10\%$  positive tumor cells with  $>2$  intensity.

### EGFR FISH

FISH assays were carried out in 2-mm cores in tissue microarrays containing representative malignant cells. The EGFR FISH assay was performed with the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysis/Abbott Molecular, Des Plaines, IL, USA), according to methods described elsewhere [13]. Four physically distant tumor areas were selected on the H&E-stained slides, and the EGFR and CEP7 signals were counted in at least 100 nuclei per tumor area at 1,000 $\times$  magnification.

## EGFR gene polymorphisms

Fifty nanograms of DNA was amplified in a 20- $\mu$ l reaction solution containing 10  $\mu$ l of 2X concentrated HotStarTaq Master Mix (Qiagen, Hilden, Germany), including polymerase chain reaction (PCR) buffer with 3 mM MgCl<sub>2</sub>, 400  $\mu$ M each dNTP, and 0.3  $\mu$ M of each primer pairs. The primer sequences specific for microsatellite marker in EGFR intron 1 were as follows: 5'-FAM-labeled forward EGFR primer; 5'-TGAAGAATTTGAGCCAACCAAA-3' and unlabeled reverse EGFR primer; 5'-CACTTGAAC CAGGGACAGCA-3'. The number of CA dinucleotide repeats of the PCR products was resolved on the GeneMapper Software v3.7. PCR product from A431 was used to confirm the number of CA dinucleotide repeats.

## KRAS, BRAF, and PI3KCA mutations

We searched for KRAS point mutations in codons 12 and 13, two hotspots that cumulatively include >95% of mutations in this gene [14]. BRAF mutations were investigated in exon 15, in which >95% of BRAF point mutations occur [15]. Exons 9 and 20 of the alpha polypeptide (the catalytic subunit of the PI3K protein [PI3KCA]), which are frequently mutated in CRC, were amplified using a standard PCR protocol, and previously described primers.

## Statistical analyses

The association between primary tumors and paired metastatic sites for IHC, EGFR gene status, EGFR intron 1 CA repeat number, and mutational status of KRAS, BRAF, and PI3KCA was evaluated by means of the Cohen's  $k$  test, appropriate for the assessment of the concordance between two categorical measurements of the same individual. A moderate and good agreement was defined as a coefficient of  $0.41 \leq \kappa \leq 0.60$  and  $0.61 \leq \kappa \leq 0.80$ , respectively.

The statistical analyses of categorical variables were performed using Pearson's  $\chi^2$  test or Fisher's exact test. Comparison of the mean between groups was calculated by Student's  $t$  test. The median duration of progression-free survival (PFS) and overall survival (OS) was calculated using the Kaplan–Meier method. Comparisons between different groups were made using the log-rank tests. Multivariate analyses were performed using a logistic regression model for response and Cox regression model for PFS and OS to identify independent factors and adjust for baseline characteristics. Two-sided  $P$  values of  $<0.05$  were considered significant. All analyses were performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No: H-0808-031-253).

## Results

### Patient characteristics and efficacy

The patient characteristics are summarized in Table 1.

**Table 1** Baseline characteristics

Characteristics	No.	%
No. of patients	75	
Age, years		
Mean	59	
Range	29–78	
Gender		
Male	47	62.7
Female	28	37.3
Available tissue		
Total tissue cases	97	
Primary site only	38	50.7
Metastasis only	15	20
Paired primary and metastatic site	22	29.3
Number of metastatic organs		
1	17	22.7
2	25	33.3
3	22	29.3
>3	11	14.6
Previous lines of chemotherapy		
0	3	4.0
1	12	16.0
2	34	45.3
>2	26	34.6
Combined therapy		
Cetuximab alone	8	10.7
Oxaliplatin-based	9	12
Irinotecan-based	56	74.7
Oral 5-FU	2	2.7
Mean duration of cetuximab (days) (range)		
Cetuximab alone	50 (7–88)	
Oxaliplatin-based cetuximab	136 (24–296)	
Irinotecan-based cetuximab	105 (7–462)	
Oral 5-FU-based cetuximab	135 (26–246)	
Performance status		
0	9	12
1	48	63
2	15	20
3	3	4.0

The median number of metastatic sites before starting treatment was 2 (range, 1–6). The most common metastatic site in these patients was the liver (52 patients [69.3%]). Thirty-five (46.7%), 36 (48%), and 24 (32%) patients had lung, lymph node, and peritoneum metastasis, respectively. All but three patients (4%) received cetuximab-containing chemotherapy as a second-line or greater treatment. In 75 patients, the number of available tissues was 97, and primary tumor only was available in 38 patients, whereas metastatic tissue only was available in 15 patients. In 22 patients, we obtained primary tumors and paired metastases.

Response was evaluable in 64 patients (85.3%). We observed 11 PRs, 27 SDs, and 26 instances of progressive disease (PD). Therefore, the overall response rate (RR) was 17.2% (95% CI, 7.7–26.7), and the disease control rate was 59.4% (95% CI, 47.0–71.7). The median duration of follow-up was 197 days (range, 16–1,022 days).

During treatment, a skin rash developed in 38 patients (50.7%). Grades 1, 2, and 3 skin toxicity were observed in 19 (25.3%), 15 (20%), and four patients (5.3%), respectively. Median PFS and OS were 122 days (95% CI, 82–161) and 246 days (95% CI, 158–333).

#### Results of molecular characteristics

The molecular characteristics are shown in Table 2.

Among 57 patients who were evaluated by FISH, EGFR gene amplification and high polysomy was observed in 5 (8.8%) and five patients (8.8%), respectively.

Sixty-eight patients were assessable for the EGFR intron 1 CA repeat polymorphism. The median sum of both alleles was 39 (range, 29–48). We defined low and high CA repeats as those presenting the sum of both allele  $\leq 36$  and  $\geq 37$ . Twenty-three patients (33.3%) showed a low CA repeat, whereas 46 (66.7%) had a high repeat.

A KRAS mutation was found in the tumor from 19 patients (27.5%) in codon 12 (14 patients) or codon 13 (five patients). A BRAF V600E mutation was observed in tumors of five patients (7.0%), and a PI3KCA mutation was detected in 1 of 57 patients (1.8%).

#### Concordance between primary tumors and paired metastases

The concordance of molecular markers between primary tumors and paired metastases is summarized in Table 3. A BRAF mutation tested on primary tissues was concordant with the results of metastases (kappa = 0.692; good strength of agreement,  $P = 0.001$ ). The EGFR intron 1 CA repeat number showed concordant expression (kappa = 0.600, moderate strength of agreement,  $P = 0.003$ ). While a KRAS mutation between primary tumors and metastases did not show statistically significant concordance (95% CI, kappa = 0.433,  $P = 0.074$ ), KRAS mutations had a tendency toward concordant expression. Other markers were discordant.

#### Comparison of characteristics between KRAS wild types and mutants

We identified 19 KRAS mutant tumors and 50 KRAS WT. There were no differences in expression of EGFR, P-EGFR positivity, PTEN positive rate, and EGFR gene amplification and high polysomy.

In KRAS WT patients, IGF-1R expression was lower (5, 11.6%) than KRAS mutants (5, 31.2%,  $P = 0.074$ ). Even though there were no BRAF mutations in KRAS mutants, it was not statistically significant ( $P = 0.311$ ; Table 4).

We detected 10 PRs (23.8%) and 1 PR (5.9%) in KRAS WTs and mutants ( $P = 0.151$ ), respectively. The median PFS of KRAS WTs and mutants was 123 (95% CI, 25–220) and 121 days (95% CI, 70–175;  $P = 0.298$ ), respectively.

**Table 2** The results of molecular characteristics

Molecular characteristics	Successfully tested cases	Positive case (%)
EGFR expression	70	45 (64.3)
p-EGFR expression	61	9 (14.8)
PTEN expression	69	35 (50.7)
IGF-1R expression	72	10 (16.1)
EGFR amplification or high polysomy	57	10 (17.6)
EGFR intron CA 1 repeat length $\geq 37$	69	46 (66.7)
KRAS mutation	69	19 (27.5)
Codon 12		14 (18.7)
Codon 13		5 (6.7)
BRAF V600E mutation	71	5 (6.7)
PI3KCA mutation	57	1 (1.3)

**Table 3** Discordance between primary tumors and metastases

Primary tumors	Negative in metastatic tissue	Positive in metastatic tissue	Concordance rate (%)	Kappa	Lower bound	Upper bound	<i>P</i> value
EGFR PharmDx (%)							
(–)	3	6	65	0.255	–0.115	0.625	0.178
(+)	1	10					
P-EGFR IHC (%)							
(–)	11	3	75	0.200	–0.326	0.726	0.383
(+)	1	1					
PTEN IHC (%)							
(–)	4	8	50	0.074	–0.284	0.432	0.690
(+)	2	6					
IGF-1R IHC (%)							
(–)	9	5	60	–0.125	–0.344	0.094	0.464
(+)	1	0					
EGFR FISH (%)							
Negative	12	1	86	0.423	–0.240	1.000	0.101
Positive	1	1					
KRAS mutation (%)							
WT	10	2	76	0.433	–0.035	0.901	0.074
Mutant	2	3					
BRAF mutation (%)							
WT	15	0	90	0.692	0.307	1.000	0.001
Mutant	2	3					
EGFR intron 1 CA repeat							
Sum $\leq 36$	6 <sup>a</sup>	0 <sup>b</sup>	80	0.600	0.279	0.921	0.003
Sum $\geq 37$	4 <sup>a</sup>	10 <sup>b</sup>					

<sup>a</sup> Sum of EGFR intron 1 CA repeat number in metastatic tissue  $\leq 36$

<sup>b</sup> Sum of EGFR intron 1 CA repeat number in metastatic tissue  $\geq 37$

The median OS in KRAS WT and mutants was 246 (95% CI, 174–317) and 258 days (95% CI, 125–390;  $P = 0.809$ ), respectively.

#### Predictive markers for treatment outcomes in KRAS WT tumors

Among 50 patients with KRAS WT tumors, responses were evaluable in 42 patients, showing a response rate of 23.8% (95% CI, 10.4–37.2%), and the disease control rate was 59.5% (95% CI, 44.0–75.0%). The first- and second-line treatment compared to greater than second-line treatment was associated with good response based on multivariate analysis (HR 0.046; 0.004–0.497,  $P = 0.011$ ). Single organ metastasis was the only meaningful factor related to higher disease control rate ( $P = 0.001$ ). Based on multivariate analysis, single organ metastasis (HR 2.425; 1.231–4.775,  $P = 0.01$ ) and development of skin toxicity (HR 0.461; 0.255–0.832) were related to a longer PFS. Skin toxicity (307 days vs. 159 days,  $P = 0.037$ ), good PS (307 days vs. 74 days,  $P < 0.001$ ), and single organ

metastasis (535 days vs. 159 days,  $P = 0.002$ ) were associated with a longer OS based on univariate and multivariate analyses.

There was no responder with a BRAF mutation ( $P = 0.563$ ). Also, the disease control rate was higher in BRAF WT patients than patients with BRAF mutations ( $P = 0.636$ ). Patients with BRAF mutations had shorter PFSs (139 days vs. 69 days,  $P = 0.068$ ) and OSs (226 days vs. 74 days,  $P = 0.908$ ; Fig. 1). In KRAS WTs, patients with high EGFR intron 1 CA repeat numbers showed a tendency for shorter PFS and OS than those with short CA repeat numbers (Fig. 1). The results of PFS and OS of KRAS WT patients are summarized in Table 5.

#### Predictive markers for treatment outcomes in KRAS mutant tumor

Among 19 patients with KRAS mutations, only one patient responded to cetuximab-containing chemotherapy. The PFS and OS in these patients were 121 days (95% CI,

**Table 4** Characteristics comparison between KRAS WT and KRAS mutants

Characteristics	No. of patients in K-ras wild type	No. of patients in K-ras mutated type	<i>P</i> value
Number of cases	50	19	
Mean age, years (range)	56 (28–77)	54 (38–76)	0.378*
Men (%)	31 (62%)	11 (57.9%)	0.755 <sup>†</sup>
ECOG PS 0-1	38 (76%)	14 (77.8%)	1.000 <sup>†</sup>
CEA <5	10 (20%)	1 (5.6%)	0.265 <sup>†</sup>
Number of metastatic organs			
Metastasis with single organ	15 (30%)	2 (10.5%)	0.124 <sup>†</sup>
Metastasis of multiple organs	35 (70%)	18 (89.5%)	
Occurrence of skin rash	26 (65%)	10 (66.7%)	0.908 <sup>†</sup>
EGFR PhamDx (%)			
(–)	17 (35.4%)	4 (23.5%)	0.547 <sup>†</sup>
(+)	31 (64.6%)	13 (76.5%)	
P-EGFR IHC (%)			
(–)	34 (82.8%)	15 (88.2%)	1.000 <sup>†</sup>
(+)	7 (17.1%)	2 (11.8%)	
PTEN IHC (%)			
(–)	27 (58.7%)	11 (64.7%)	0.776 <sup>†</sup>
(+)	19 (41.3%)	6 (35.3%)	
IGF-1R IHC (%)			
(–)	38 (88.4%)	11 (68.8%)	0.074 <sup>†</sup>
(+)	5 (11.6%)	5 (31.2%)	
EGFR FISH (%)			
Negative	31 (81.6%)	12 (80%)	1.000 <sup>†</sup>
Positive	7 (18.4%)	3 (20%)	
EGFR intron 1 CA repeat			
Sum ≤36	14	9	0.127 <sup>†</sup>
Sum ≥37	33	9	
BRAF mutation (%)			
WT	42 (89.4%)	19 (100%)	0.311 <sup>†</sup>
Mutatnt	5 (10.6%)	0	

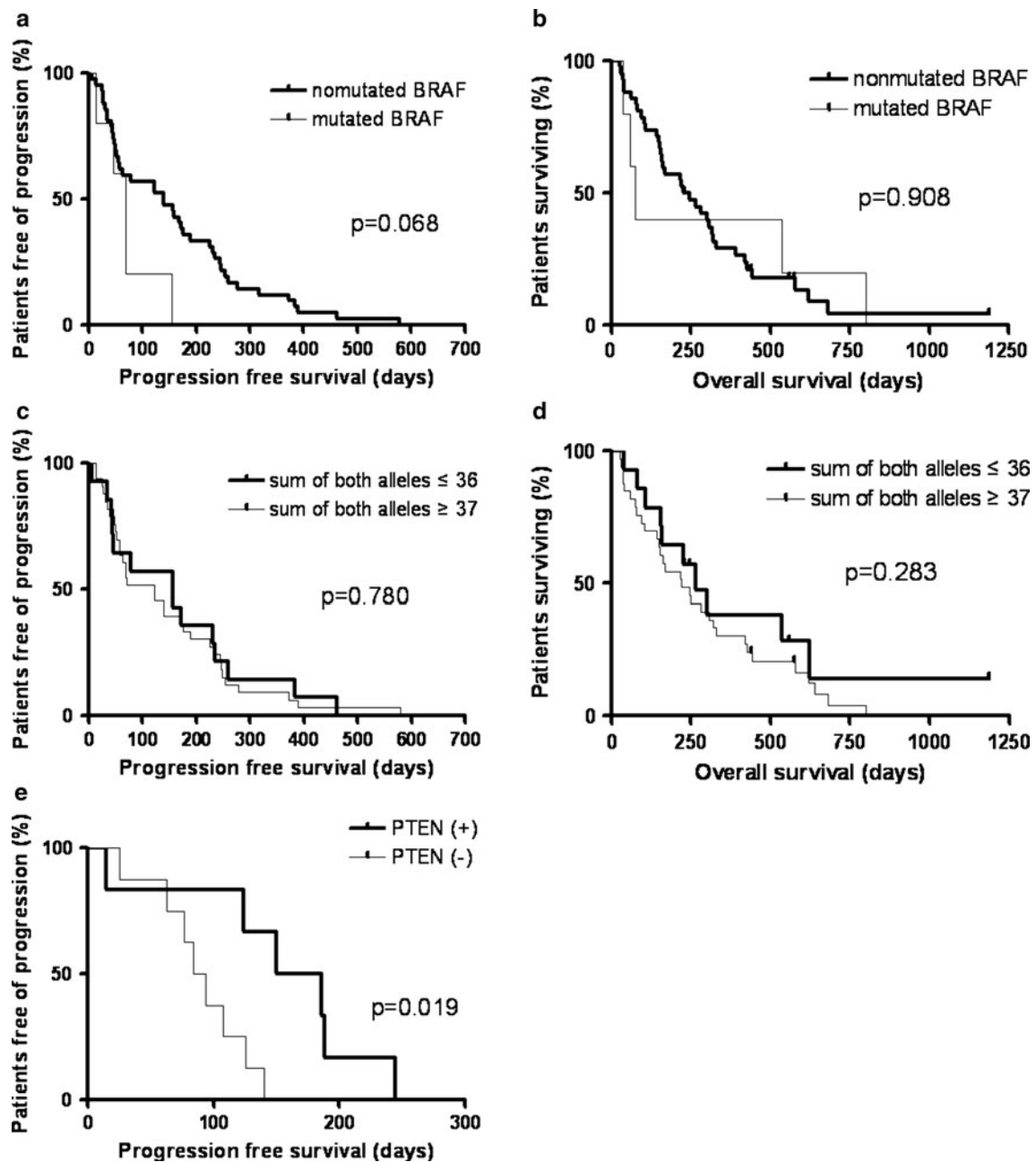
\* Based on Student's *t* test<sup>†</sup> Based on Fisher's exact test

78–163) and 258 days (95% CI, 125–390). Based on univariate analysis, PTEN expression in primary tumors was associated with a longer PFS, with a median PFS of 150 days (95% CI, 75–224) compared to 84 days in tumors that did not express PTEN (95% CI, 60–107; *P* = 0.019; Fig. 1). Additionally, development of skin toxicity was also a predictive marker of a favorable PFS (140 days vs. 77 days, *P* = 0.002). Based on multivariate analysis, expression of PTEN correlated with improved PFS (HR 0.141; 0.023–0.857, *P* = 0.033), and skin toxicity was a predictive marker for a longer PFS (HR 0.243; 0.059–1.003, *P* = 0.050). In addition, there was a trend toward a longer PFS in patients with a short EGFR intron 1 CA repeat number, but the difference did not reach the threshold of significance (126 days vs. 94 days, *P* = 0.704).

## Discussion

This study can give us the information of discrepancy of molecular markers between primary tumor tissue and metastatic tumor tissue, and more detailed predictive markers for obtaining benefit from cetuximab therapy beyond KRAS.

It is widely accepted that EGFR expression does not predict benefit from cetuximab treatment as was the same in our study, and the role of pEGFR should be validated in more studies even though our study did not support its predictive role. Loss of PTEN by IHC or loss of PTEN gene function was reported as one of the predictive markers [16]. In patients who were treated with cetuximab plus irinotecan and did not have KRAS mutations in primary tumors, PTEN loss in metastases was reported to be



**Fig. 1** Kaplan–Meier curve for progression-free survival and overall survival according to molecular character. **a** Progression-free survival according to BRAF mutation in KRAS WT. **b** Overall survival according to BRAF mutation in KRAS WT. **c** Progression-free survival

according to EGFR intron 1 CA repeat number in KRAS WT. **d** Overall survival according to EGFR intron 1 CA repeat number in KRAS WT. **e** Progression-free survival according to PTEN expression of primary sites in KRAS mutant

predictive of resistance to cetuximab [11]. On the other hand, in KRAS mutants, our work suggested that PTEN loss could be a predictive marker for poor outcome.

Although it has been hypothesized that PIK3CA mutations may be a predictive marker for poor response to cetuximab treatment [6, 17], several data, including our results, showed no correlation between PI3KCA mutations and the response to cetuximab treatment [18]. The mutation rate of PIK3CA in colon cancer was reported to be

7–18% [19, 20]; we had only one patient with a PIK3CA mutation. Interestingly, a PIK3CA mutant also presented with a KRAS mutation simultaneously. Also, we recognized the tendency of poor outcomes in BRAF mutants, although it did not reach the threshold of significance because of the small sample size.

There have been some concerns that EGFR FISH is not a standardized and easily reproducible method, and EGFR gene copy number could reflect EGFR activity [21, 22].

**Table 5** Analysis of PFS and OS in KRAS WT patients

Variable	Patients with KRAS WT ( <i>n</i> = 50)		<i>P</i> value
	No.		
Responder (response rate, %)	10 (23.8%)		
Median PFS, days (95% CI)	123 (25–220)		
Median OS, days (95% CI)	246 (174–317)		
	Non-mutated BRAF ( <i>n</i> = 34)	Mutated BRAF ( <i>n</i> = 5)	
Responder (response rate, %)	8 (23.5)	0 (0)	0.563*
Median PFS, days (95% CI)	139 (55–222)	139 (55–222)	0.068†
Median OS, days (95% CI)	226 (106–345)	74 (46–101)	0.983†
	Development of skin toxicity ( <i>n</i> = 17)	No skin toxicity ( <i>n</i> = 25)	
Responder (response rate, %)	9 (36%)	1 (5.9%)	0.031*
Median PFS, days (95% CI)	176 (82–269)	64 (33–94)	0.009†
Median OS, days (95% CI)	307 (230–383)	159 (83–234)	0.037†
	Single organ metastasis ( <i>n</i> = 11)	Multiple organ metastases ( <i>n</i> = 31)	
Responder (response rate, %)	4 (36.4)	6 (19.4)	0.410*
Median PFS, days (95% CI)	235 (135–334)	64 (41–86)	0.008†
Median OS, days (95% CI)	535 (250–819)	159 (72–245)	0.002†
	PS 0-1 ( <i>n</i> = 32)	PS 2-3 ( <i>n</i> = 10)	
Responder (response rate, %)	9 (28.1)	1 (10)	0.404*
Median PFS, days (95% CI)	140 (85–194)	53 (0–110)	0.028†
Median OS, days (95% CI)	307 (246–367)	74 (0–209)	<0.001†
	Short CA repeat number ( <i>n</i> = 10)	High CA repeat number ( <i>n</i> = 29)	
Responder (response rate, %)	3 (30)	6 (20.7)	0.669*
Median PFS, days (95% CI)	156 (12–299)	122 (43–200)	0.780†
Median OS, days (95% CI)	266 (159–372)	221 (124–317)	0.283†
	EGFR FISH-negative ( <i>n</i> = 26)	EGFR FISH-positive ( <i>n</i> = 6)	
Responder (response rate, %)	6 (23.1)	2 (33.1)	0.625*
Median PFS, days (95% CI)	70 (0–159)	173 (80–265)	0.584†
Median OS, days (95% CI)	221 (84–357)	217 (76–357)	0.910†

PFS progression-free survival, OS overall survival

\* Based on Fisher's exact test

† Based on log-rank test

Our results showed that the EGFR amplification by FISH in KRAS WT could be a good predictive marker for treatment outcome of cetuximab, though it was not statistically significant.

Interestingly, we found the concordant expression of EGFR intron 1 CA repeat number between primary tumor and metastasis. It has been hypothesized that the number of CA single sequence repeat in the intron 1 of EGFR gene, which affects transcription efficiency of the genes, is associated with the response to EGFR inhibitors [23]. Previous report suggested that in a clinical study in colorectal cancer, polymorphic variations in the intron 1 of the EGFR gene are associated with response to EGFR inhibitors and may provide an explanation as to why the development of skin toxicity is associated with a favorable outcome [10]. Patients presenting high CA

repeats, regardless of mutational status of KRAS, had a shorter PFS, which is similar to a previous report [24]. Our data added to growing evidence that EGFR intron 1 CA repeat number may be a predictive marker for cetuximab treatment outcome. These data warrant additional large-scale clinical studies to validate and integrate EGFR genotyping as a marker for the selection of patients to be treated with cetuximab.

So far, molecular marker studies of cetuximab have been mostly evaluated in primary tumors. Among several studies that investigated primary tumors and paired metastases by IHC, some studies have shown discordance of EGFR expression [2], and there was another study showing concordance of EGFR IHC [25]. We observed a different EGFR expression in primary tumors and metastases and suggest a genetic alteration in metastases.



KRAS mutations were found in 19 patients (27.6%) in this study, which was lower than that of previously reported data. Several considerations might be given to this point. First, technical problems could be the reason. Second, this might be explained by ethnic differences. One report suggested KRAS mutation rates are higher among African American than Caucasian [26]. Whereas majority of studies about KRAS mutation were done in Western country, there was no data about KRAS mutation rates in Asian population. Our study population consisted of Asian population. For this issue, more studies with large number of patients should be further needed. Third, this may reflect discordance of KRAS mutation between primary sites and metastases. While many previous studies had assessed the mutation status of KRAS in primary sites, our study included results from metastatic site if there was not available primary tumor tissue.

In case of KRAS mutation, a high concordance rate of 90% between primary tumors and metastases has been reported [27, 28]. But in our data, the concordance rate of KRAS mutation was lower (76%) than previous reports, and there was marginal statistical significance in the concordant expression of KRAS mutations between primary tumors and metastases. We suggest a change in the carcinogenesis pathway and the requirement of rebiopsy before cetuximab treatment is taken into consideration.

There is one previous report about differences in BRAF mutations. According to the results, one patient out of two with BRAF mutations in primary tumors showed a BRAF WT in metastasis [29]. We evaluated 20 patients with primary tumors and metastases and showed a high concordance rate (up to 90%) that was statistically significant.

All other molecular markers except the EGFR intron 1 CA repeat number and BRAF mutation were discordant between the primary site and metastasis.

These were meaningful results because there have been no published data regarding a discordance between these markers in mCRC.

In addition, our data showed a different expression rate of IGF-1R based on KRAS mutational status. In KRAS mutants, IGF-1R expression was observed more frequently than in KRAS WT patients. Previous reports have suggested the activation of IGF1/IGF1-R involved in transformation and tumorigenicity in colon cancer, and there is a tendency for a stronger expression of IGF-1R in larger colon tumors and in the presence of metastatic disease [7, 8]. Although we did not show differences in treatment outcome according to IGF-1R expression, it would be worthwhile to evaluate the functional association between both of them.

We confirmed that skin toxicity is a strong predictive marker for cetuximab treatment in mCRC, as previous studies have proved [30].

Our study was performed retrospectively in a small population; this is one of limitation of our study. Furthermore, enrolled patients were heterogenous. The majority of our population (80%) was treated with two or more chemotherapy regimens before cetuximab treatment. The benefit of cetuximab added to first-line or second-line irinotecan containing therapy has been addressed in two trials: the CRYSTAL and OPUS trials [30, 31]. However, our data provided some valuable information for the clinician that cetuximab has efficacy in heavily pre-treated patients with KRAS WTs. Furthermore, we demonstrated expression of variable molecular markers which could be predictive for treatment outcome of cetuximab therapy in mCRC. There have been few results about the association between EGFR intron 1 CA repeat length and outcome. The results that we obtained in primary tumors and paired metastases showed discordant expression of the majority of molecular markers. Furthermore, there have been few studies that have been performed in Eastern countries. Therefore, our data could provide a reference of expression for molecular markers in the Asian population, simultaneously giving information about who has a higher possibility to get benefits from cetuximab therapy before starting treatment.

We conclude that a skin rash is strong predictive marker, regardless of molecular markers, for good outcome in mCRC patients receiving cetuximab chemotherapy. And all other molecular markers except the EGFR intron 1 CA repeat number and BRAF mutation were discordant between the primary tumor and metastasis. In KRAS mutants, PTEN expression by IHC in the primary site could be a promising predictive marker, and in KRAS WT patients, a BRAF mutation was a strong predictive marker for poor treatment outcome. It is possible that EGFR intron 1 CA short repeat number and EGFR amplification are associated with favorable outcome in KRAS WT.

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