Association between polymorphisms in EGFR and tumor response during cetuximab and oxaliplatin-based combination therapy in metastatic colorectal cancer: Analysis of data from two clinical trials

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Abstract. Predicting tumor response prior to starting anti-epidermal growth factor receptor (EGFR) antibody therapy would benefit patients with advanced/metastatic colorectal cancer (mCRC). The present study investigated the association between efficacy of cetuximab treatment and gene polymorphisms of fragment C γ receptor (FcγR) 2A, FcγR3A and EGFR in patients with extended RAS/BRAF wild-type mCRC. Clinical data and specimens were obtained from 90 patients who participated in either of two clinical studies evaluating the first-line, cetuximab plus oxaliplatin-based treatment. It was hypothesized that polymorphisms H/H of FcγR2A, V/V of FcγR3A, K/K of EGFR and <36 CA repeats in the EGFR gene may be associated with a favorable tumor response. Multivariate analysis demonstrated that patients with the H/H polymorphism tended to have an improved tumor response compared with the non-H/H population, although the result was not significant [odds ratio, 2.25; 95% confidence interval (CI), 0.89-5.66; P=0.09]. Univariate analysis revealed increased tumor shrinkage in patients with the K/K polymorphism of EGFR compared with the other polymorphisms (mean ± standard deviation, -55.3±28.4 vs. -39.6±40.8%; P=0.04). Subsequent multivariate analysis confirmed that the K/K polymorphism of EGFR predicted greater tumor shrinkage (multiple linear regression analysis estimate, -19.3; 95% CI, -35.5 to 3.0; P=0.02), with the tendency toward a preferable response in patients with <36 CA EGFR gene repeats (estimate, -16.9; 95% CI; -34.4 to 0.6; P=0.06). However, other polymorphisms and clinical variables did not predict tumor shrinkage. In conclusion, the present study demonstrated that polymorphisms of EGFR, FcγR2A and FcγR3A may differentiate the patients that obtain the maximum benefit from cetuximab treatment.

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Introduction

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase found on the cell surface that is often upregulated in tumor cells (1). EGFR plays a pivotal role in cell proliferation by activating downstream signaling pathways (2). Cetuximab, a chimeric immunoglobulin G1 monoclonal antibody against EGFR, blocks the function of EGFR by competitively antagonizing and/or internalizing the receptor (3,4). Clinical studies have demonstrated its efficacy in the treatment of a number of different types of cancer, including advanced and metastatic colorectal cancer (mCRC) (1,4).

Signaling from EGFR is relayed by a GTPase transducer protein named RAS, and RAS-associated mutations in tumor

cells are associated with resistance to cetuximab treatment (5,6). Therefore, the clinical use of cetuximab is limited to patients with RAS wild-type mCRC. In addition, RAS is not a sufficient biomarker for predicting tumor response, and disease control is observed in only half of patients with KRAS wild-type mCRC subjected to monotherapy as first- or later-line treatment (7,8). Therefore, additional predictors of tumor response to cetuximab are required in order to avoid poor treatment efficacy with unnecessary adverse reactions.

Antibody-dependent, cell-mediated cytotoxicity (ADCC) is proposed as a distinct mechanism of antitumor activity by cetuximab, and thus has gathered attention as a potential predictor of treatment efficacy and/or safety (9,10). Cetuximab has an antigen-binding and crystalline fragment (Fc fragment) in its structure (9,11) allowing it to bind to both the tumor antigen (EGFR) and fragment C γ receptor (FcγR) located on immune cells, and to trigger ADCC (11). A histidine (H)/arginine (R) polymorphism at position 131 on FcγR2A and a valine (V)/phenylalanine (F) polymorphism at position 158 on FcγR3A are associated with different affinities for human IgG (12). According to the accumulating evidence, patients harboring FcγR2A-131H/H and FcγR3A-158V/V mutations are expected to have stronger ADCC during monoclonal antibody therapies (13-15).

Furthermore, higher EGFR expression levels due to lower numbers of CA repeats in EGFR intron 1 may increase the response to cetuximab (16,17). In addition, a substitution from R to lysine (K) in codon 521 of the extracellular domain of EGFR could result in lower ligand binding affinity, downregulation of the target gene, and consequent favorable response to cetuximab treatment (18,19). Despite these promising findings, clinical studies remain scarce, and interpretations of the results are conflicting due to several limiting factors. The present study thus investigated the association between gene polymorphisms in FcγR2A, FcγR3A and EGFR and the efficacy of first-line cetuximab and oxaliplatin treatment in patients with extended RAS/BRAF wild-type mCRC.

Materials and methods

Patients. The present study reviewed the clinical data of patients participating in one of two trials evaluating the efficacy of combination therapy with cetuximab and oxaliplatin-based chemotherapy as a first-line treatment (UMIN 000003253 and UMIN000007195) (20,21). The patients were recruited, and the specimen was collected from 31 institutes in Japan between April 2010 and May 2011, and between February, 2012 and February, 2013. These institutes included Chiba Cancer Center (Chiba, Japan); Fukui-Ken Saiseikai Hospital (Fukui, Japan); Gifu University Hospital (Gifu, Japan); Hokkaido Cancer Center (Sapporo, Japan); Ishikawa Prefectural Central Hospital (Kanazawa, Japan); Japan Community Health Care Organization (JCHO) Osaka Hospital (Osaka, Japan); Kagawa University Hospital (Kita, Japan); Kanagawa Cancer Center (Yokohama, Japan); Kanazawa Medical University Hospital (Kahoku, Japan); Kansai Medical University Hospital (Hirakata, Japan); Kitakyushu General Hospital (Kitakyushu, Japan); Kobe Ekisaikai Hospital (Kobe, Japan); Kochi Medical School Hospital (Nankoku, Japan); Matsunami General Hospital (Hashima, Japan);

Nakadori General Hospital (Akita, Japan); National Hospital Organization Nagoya Medical Center (Nagoya, Japan); National Hospital Organization Osaka National Hospital (Osaka, Japan); Osaka City University Graduate School and Faculty of Medicine (Osaka, Japan); Osaka General Medical Center (Osaka, Japan); Osaka Rosai Hospital (Sakai, Japan); Osakakita Teishin Hospital (Osaka, Japan); Rinku General Medical Center (Izumisano, Japan); Sakai City Medical Center (Sakai, Japan); Sano Hospital (Kobe, Japan); Showa University Fujigaoka Hospital (Yokohama, Japan); Teikyo University Chiba Medical Center (Ichihara, Japan); Toyama Prefectural Central Hospital (Toyama, Japan); University of Occupational and Environmental Health (Kitakyushu, Japan); Toyonaka Municipal Hospital (Toyonaka, Japan); Yamaguchi University Hospital (Ube, Japan); and Yokoyama Hospital for Gastroenterological Diseases (Nagoya, Japan). The primary endpoint of these two trials was response rates (RRs) with confirmation, as evaluated by computed tomography at 4- to 8-weekly intervals. The RR in the present study was regarded as the clinically important primary endpoint as per the previous two clinical trials (20,21). In total, 90 patients, with RAS/BRAF wild-type mCRC were identified, and the polymorphisms of these patients were analyzed. The mean age of the patients was 66.3±9.9 years (standard deviation). The present study was approved by the Institutional Review Board of Yamaguchi University School of Medicine (approval number, H28-171) and performed in accordance with the Declaration of Helsinki. The requirement for informed consent was waived as the present study was a retrospective analysis of previously collected samples and data. The patients were given the opportunity to refuse the use of their samples in the present study, according to the ethics guidelines of the Institutional Review Board. Formalin-fixed, paraffin-embedded (FFPE) samples collected as part of the previous studies were used to analyze polymorphisms in FcγR2A, FcγR3A and EGFR for the present study. The data used in the present study was collected by Case Report Form for each clinical trial. No additional data/samples were collected for the present study.

Evaluation of polymorphisms in FcγR2A, FcγR3A and EGFR. DNA was extracted from the FFPE samples. Applying micro-dissection on 10 μm sections, non-tumor tissues were dissected from the FFPE samples. DNA extraction was performed using a QIAamp DNA FFPE Tissue kit (Qiagen GmbH) according to the manufacturer's protocol. The TaqMan technique was then used to determine FcγR2A-H131R rs1801274, FcγR3A-V158F rs396991 and EGFR-R521K rs2227983 polymorphisms using established primers (22), TaqMan SNP Genotyping Assays C_9077561_20, C_25815666_10 and C_16170352_20 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the TaqMan Genotyping Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). In brief, a 5-μl reaction solution, containing TaqMan Genotyping Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), Assay Mix, and 20-40 ng of genomic DNA diluted in dH$_2$O, was incubated in 384-well microtiter plates at 50°C for 2 min to degrade dU-containing DNA, followed by incubation at 95°C for 10 min (denaturation), followed by 40 cycles of 15 sec at 95°C and 1 min of annealing and extension at 60°C. The ABI Prism 7900HT (Applied Biosystems;

Table I. Clinical characteristics and frequencies of polymorphisms.

Variables	Number of patients (%)
Sex	
Female	34 (37.8)
Male	56 (62.2)
ECOG-PS	
0	79 (87.8)
1	11 (12.2)
Treatment	
FOLFOX	37 (41.1)
CapeOX	53 (58.9)
Primary tumor site	
Colon	51 (56.7)
Rectum	39 (43.3)
FcγR2A (H131R)	
H/H	55 (61.1)
H/R	31 (34.4)
R/R	3 (3.3)
Not determined	1 (1.1)
FcγR3A (V158F)	
V/V	12 (13.3)
V/F	35 (38.9)
F/F	43 (47.8)
EGFR (R521K)	
K/K	33 (36.7)
K/R	39 (43.3)
R/R	18 (20.0)
EGFR (CA repeat)	
<36	25 (27.8)
≥36	65 (72.2)

ECOG-PS, Eastern Cooperative Oncology Group Performance Status; FOLFOX, FOLFOX + cetuximab treatment, CapeOX, CapeOX + cetuximab treatment; EGFR, epidermal growth factor receptor; FcγR, fragment C γ receptor; H, histidine; V, valine; K, lysine; R, arginine; F, phenylalanine.

Thermo Fisher Scientific, Inc.) was used for end-point reading of the fluorescence generated during PCR amplification. EGFR CA Repeats in Intron 1 Genotyping was determined via direct sequencing, as previously described (23,24).

Statistical analysis. The primary endpoint was RR and the secondary endpoint was the maximum change in tumor diameter from baseline, calculated using the formula (tumor diameter at evaluation-tumor diameter at baseline)/tumor diameter at baseline x100, whereby negative numbers indicate tumor shrinkage during the treatment and positive numbers indicate tumor enlargement.

The Eastern Cooperative Oncology Group Performance Status (ECOG-PS) (25), combined chemotherapy, patient sex and primary tumor sites were used as variables that may potentially affect treatment efficacy. The detailed information regarding tumor location in the colon (i.e. left- or right-side colon) was not collected in the previous clinical trials and was therefore unavailable in the present study. In order to analyze the association between the tumor response and variables, χ^2 test was performed, followed by logistic regression analysis. For the association between tumor shrinkage and variables, Welch's t-test was performed, followed by multiple linear regression analysis. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses and graph depiction were performed using Microsoft Excel (version 2013; Microsoft Corporation) and KaleidaGraph 4.5 (version 4.5; Synergy Software). The final figures were created using Photoshop CS2 (Adobe Systems).

Results

Frequency of polymorphisms and mutation status. H/H in FcγR2A was the most frequent genetic polymorphism observed in the present study (61.1%), while V/V in FcγR3A was observed in only 12 patients (13.3%) (Table I). K/K in EGFR was observed in 33 patients (36.7%), while 27.8% of the patients had <36 CA repeats in EGFR. In one patient, FcγR2A polymorphisms could not be determined due to DNA fragmentation, and their data concerning FcγR2A were excluded from further analysis.

In terms of the mutation status, one of the two clinical trials only recruited patients with KRAS wild-type CRC. Therefore, the total rate of RAS/BRAF mutations could not be assessed in this retrospective study.

RR. The univariate analysis demonstrated no significant difference in RR between patients with and without the tested polymorphisms (Table II). Therefore, the odds ratio for tumor response was estimated using all listed variables (Fig. 1). The patients with an H/H polymorphism in FcγR2A (vs. non-H/H polymorphism) had an odds ratio of 2.25, although this was not statistically significant (P=0.09).

Maximum change in tumor diameter from baseline. The maximum change in tumor diameter from baseline was used as a secondary endpoint in the present study to investigate the influence of gene polymorphisms. As the present study included patients who had received cetuximab in addition to conventional cytotoxic chemotherapy, the maximum change in tumor diameter from baseline (continuous scale) was considered to be a more sensitive endpoint for measuring the association between polymorphisms and treatment efficacy. As the tumor diameter information was unavailable for one patient, the analyses were performed using the data of 89 patients.

Notably, patients with a K/K polymorphism in the EGFR gene exhibited greater tumor shrinkage compared with the patients with K/R or R/R in the EGFR gene (P=0.04; Fig. 2). The multivariate analysis demonstrated a significant association between the K/K polymorphism in EGFR and tumor shrinkage [multiple linear regression analysis estimate, -19.3; 95% confidence interval (CI), -35.5 to 3.0; P=0.02] (Table III). Patients with <36 CA repeats in the EGFR gene exhibited a tendency toward a better tumor response (estimate, -16.9; 95%

Table II. Univariate analysis for response rate.

Variables	CR or PR, n	RR, %	χ^2	P-value
Sex			0.48	0.49
Female	20	58.8		
Male	37	66.1		
ECOG-PS			0.42	0.52
0	51	64.6		
1	6	54.5		
Treatment			0.06	0.80
FOLFOX	24	64.9		
CapeOX	33	62.3		
Primary tumor site			0.33	0.57
Colon	31	60.8		
Rectum	26	66.7		
FcγR2A (H131R)			2.95	0.09
H/H	39	70.9		
Non-H/H	18	52.9		
FcγR3A (V158F)			0.07	0.80
V/V	8	66.7		
Non-V/V	49	62.8		
EGFR (R521K)			0.25	0.62
K/K	22	66.7		
Non-K/K	35	61.4		
EGFR (CA repeat)			0.32	0.57
<36	17	68.0		
≥36	40	61.5		

CR, complete response; PR, partial response; RR, response rate; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; FOLFOX, FOLFOX + cetuximab treatment, CapeOX, CapeOX + cetuximab treatment; EGFR, epidermal growth factor; FcγR, fragment C γ receptor; H, histidine; V, valine; K, lysine; R, arginine; F, phenylalanine.
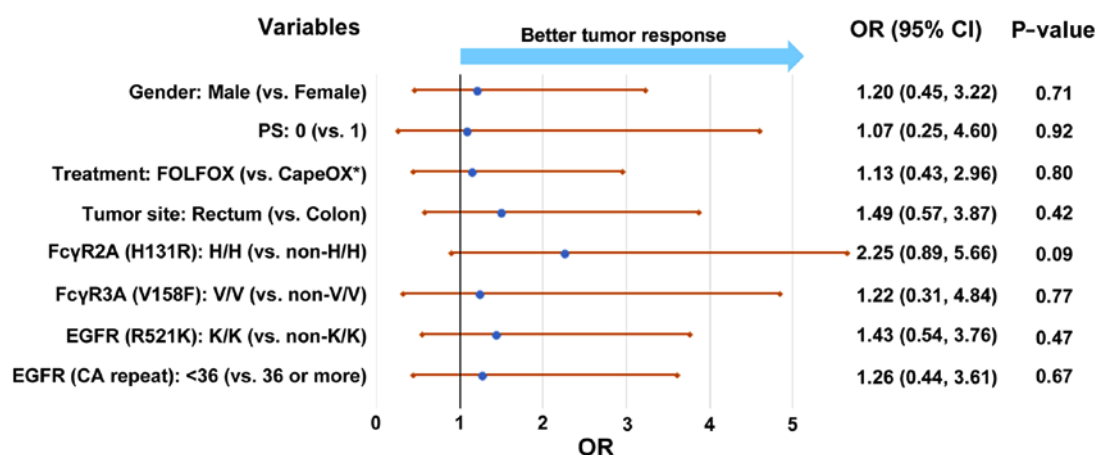

Figure 1. OR for response rate. A value of >1 suggested that the patient groups were more likely to exhibit a complete or partial response compared with the counterparts. No significant difference was observed. OR, odds ratio; CI, confidence interval; EGFR, epidermal growth factor receptor; FcγR, fragment C γ receptor; H, histidine; V, valine; K, lysine; PS, performance status.

CI, -34.4 to 0.6; P=0.06). Tumor size change at individual level was also compared between patients with and without the K/K EGFR polymorphism (Fig. 3), and was consistent with the univariate analysis in that those with the polymorphism exhibited greater tumor shrinkage than those without the gene change. However, it should be noted that there were certain

Variables	n	Mean (95%CI)		P-value
Gender				
Male	55	-44.7 (-54.8, -34.6)		0.83
Female	34	-46.5 (-59.7, -33.3)		
PS				
0	78	-46.4 (-54.6, -38.2)		0.59
1	11	-38.6 (-68.7, -8.4)		
Treatment				
FOLFOX	36	-51.1 (-64.3, -37.8)		0.25
CapeOX	53	-41.5 (-51.4, -31.6)		
Primary tumor site				
Colon	50	-43.2 (-53.4, -32.9)		0.53
Rectum	39	-48.3 (-60.9, -35.6)		
FcγR2A (H131R)				
H/H	54	-48.7 (-58.6, -38.9)		0.43
Non-H/H	34	-42.2 (-55.5, -28.9)		
FcγR3A (V158F)				
V/V	12	-47.0 (-73.8, -20.2)		0.89
Non-V/V	77	-45.1 (-53.5, -36.8)		
EGFR (R521K)				
K/K	33	-55.3 (-65.3, -45.2)		0.04
Non-K/K	56	-39.6 (-50.5, -28.7)		
EGFR (CA repeat)				
<36	24	-54.0 (-67.1, -41.0)		0.14
36 or more	65	-43.1 (-51.9, -32.5)		

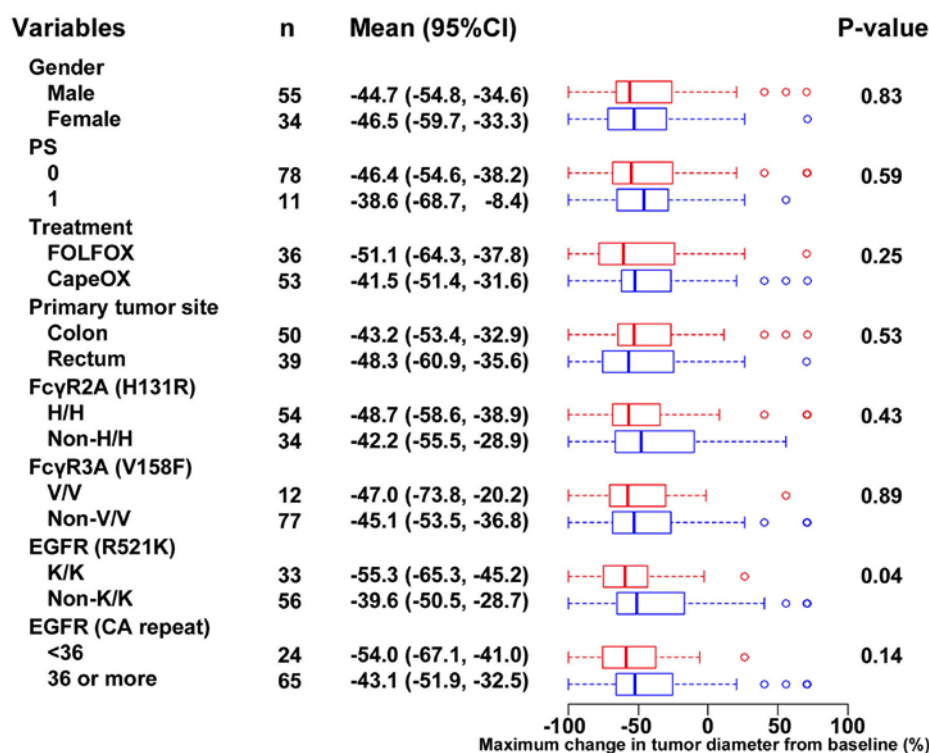Maximum change in tumor diameter from baseline (%)

Figure 2. Degree of tumor size change and univariate analysis. Maximum changes in tumor diameter from baseline is presented according to the variables. Negative numbers indicate tumor shrinkage, while positive numbers indicate tumor enlargement. Enhanced tumor shrinkage was observed among the patients with K/K polymorphisms in EGFR (R521K) compared with the patients with no K/K polymorphism. EGFR, epidermal growth factor receptor; CI, confidence interval; FcγR, fragment C γ receptor; H, histidine; V, valine; K, lysine; R, arginine; F, phenylalanine; PS, performance status.

patients that exhibited sufficient tumor shrinkage among patients with K/R or R/R in the EGFR gene.

Discussion

Previous studies investigating the association between polymorphisms of FcγR and cetuximab treatment had several limitations. First, only mCRC harboring KRAS exon2 mutations were excluded (10,26,27), and thus the influence of other mutations, such as extended RAS and BRAF, could not be ruled out. Secondly, a number of studies analyzed the combined data of patients with substantially divergent backgrounds, including line of the treatment, backbone of the chemotherapy (oxaliplatin, irinotecan or monotherapy) and even monotherapy (9,28). As discussed by Inoue *et al* (22), the deteriorated systemic and local immune systems in heavily treated patients could possibly exert only limited antitumor activity mediated by ADCC; analyzing these data without considering these factors may have led to conflicting results. In contrast, the uniquely valuable characteristic features of the present study are the exclusion of patients with mCRC that exhibited BRAF or extended RAS mutations, the inclusion of only first-line treatment regimens, and limiting the backbone treatment to oxaliplatin and fluoropyrimidines.

Under these conditions, two results were revealed: i) A clear association between the K/K polymorphism of EGFR and maximum tumor shrinkage from baseline; and ii) a tendency toward greater efficacy in tumors carrying the H/H polymorphism of FcγR2A. The former result is partly consistent with previous suggestions of an improved prognosis in patients with

the K/K polymorphism (18,19), including the observation that tumors harboring K/K or K/R exhibited favorable tumor characteristics and a higher RR to cetuximab combined chemotherapy in 112 patients with KRAS wild-type colorectal carcinoma (18). Such a result could reflect attenuated EGFR signaling and the higher sensitivity to signaling blockade by cetuximab in patients with the R521K polymorphism (18). Unlike colorectal cancer, expression of the K-allele in head and neck cancers has been associated with shorter progression free survival (PFS) and resistance to cetuximab, with stronger treatment required to induce K-alleles in ADCC cells *in vitro*, due to lower affinity (29). Although no clear explanation has yet emerged for these inconsistent observations, differing dependencies on EGFR signaling among different tumor types and different degrees of required antibody affinity for signal inhibition by cetuximab are both possible underlying mechanisms (29). Further studies are required in order to elucidate these aspects.

In contrast to the tumor shrinkage effects, polymorphisms of FcγR2A, FcγR3A and EGFR had no statistically significant association with tumor response.. Nevertheless, the multivariate analysis demonstrated a tendency for an improved tumor response in H/H tumors compared with non-H/H tumors. Specifically, a H/R polymorphism at position 131 on FcγR2A was associated with enhanced affinities for human IgG, and patients harboring FcγR2A-131H/H mutations were predicted to have stronger ADCC (13-15). A study using the data and samples from patients receiving cetuximab monotherapy for colorectal cancer demonstrated a significant association between efficacy of late-line cetuximab monotherapy and an H/H polymorphism in FcγR2A (10). The present study therefore investigated

Table III. Multiple linear regression analysis for maximum tumor change from baseline.

Variables	Groups	Estimate (SE)	95% CI	P-value
Sex	Male vs. female	1.6 (8.7)	-15.4, 18.6	0.85
ECOG-PS	1 vs. 0	-0.8 (12.6)	-25.7, 24.2	0.95
Treatment	FOLFOX vs. CapeOX	-10 (8.2)	-26.4, 6.3	0.23
Primary tumor site	Colon vs. rectum	6.5 (8.1)	-9.7, 22.6	0.43
FcγR2A (H131R)	H/H vs. non-H/H	-6.2 (8)	-22.1, 9.7	0.44
FcγR3A (V158F)	V/V vs. non-V/V	-6.1 (11.6)	-29.2, 17	0.60
EGFR (R521K)	K/K vs. non-K/K	-19.3 (8.2)	-35.5, -3.0	0.02
EGFR (CA repeat)	<36 vs. ≥36	-16.9 (8.8)	-34.4, 0.6	0.06

ECOG-PS, Eastern Cooperative Oncology Group Performance Status; FOLFOX, FOLFOX + cetuximab treatment; CapeOX, CapeOX + cetuximab treatment; SE, standard error; CI, confidence interval; EGFR, epidermal growth factor; FcγR, fragment C γ receptor; H, histidine; V, valine; K, lysine; R, arginine; F, phenylalanine.
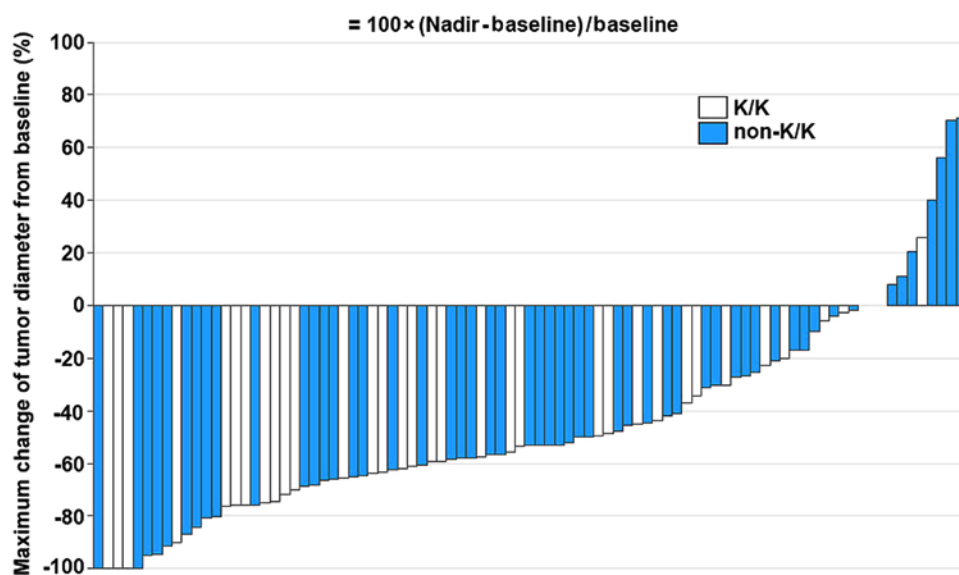

Figure 3. Waterfall plots presenting the change in tumor size in 89 patients. The individual data for tumor size is presented in white for the patients with a K/K polymorphism in EGFR (R521K) and in blue for the patients with no K/K polymorphism. EGFR, epidermal growth factor receptor; K, lysine; R, arginine.

whether combination oxaliplatin-based chemotherapy could obscure the association between tested polymorphisms and RR, as cytotoxic-doublet treatment is generally effective in 50% of patients with mCRC. In addition, a number of the patients recruited in the clinical trials assessed during the present study received a hepatectomy with curative intent, which would significantly influence PFS and overall survival.

The incidence of H/H polymorphisms in FcγR2A and V/V in FcγR3A in the present study was 61 and 13%, respectively. A previous study demonstrated that the incidence of H/H in FcγR2A was higher among Japanese patients than patients from Europe and the USA (9), and 61% in the present study is consistent with this and other previous study (9,22). In contrast, a 4-9% incidence rate of the V/V polymorphism in FcγR3A has been demonstrated (9,21), suggesting a slightly higher incidence of V/V in FcγR3A in the present study; however, the frequency of polymorphisms of certain gene differs within Japan (30), and such variability could account for the small differences in incidence between previous studies and the

present study. Although external validation was not performed in the present study, the similarities in polymorphism frequencies and the use of an established primers (22) and methods described above ensure reliability in the laboratory evaluations.

A limitation of the present study was the potential effect of polymorphisms on the treatment efficacy of cytotoxic agents, such as oxaliplatin and fluoropyrimidines. Although this is an unlikely outcome, the issue may be overcome by setting FOLFOX/CapeOX alone as a control arm and demonstrating the lack of polymorphism effects in this control group. Analyzing the data of patients receiving cetuximab monotherapy would be an alternative resolution, although cetuximab monotherapy is rarely utilized as a first- or second-line treatment. Instead, monotherapy is often used only in patients with deteriorated general status (31) or as a later treatment option, and in both these cases the immune system and ADCC may not function as expected. Another limitation was the lack of information regarding the sidedness of the primary tumors. As sidedness of the primary tumor is recognized as being significantly

associated with the efficacy of anti-EGFR therapy (32), adding sidedness to the other clinical data may further clarify the impact of polymorphisms.

In conclusion, the present study provides preliminary evidence suggesting an association between treatment efficacy and polymorphisms in the EGFR gene in patients with RAS/BRAF wild-type mCRC. Individuals harboring the K/K polymorphism in EGFR demonstrated significantly greater tumor shrinkage during treatment than those with the non-K/K polymorphism. Further studies with an appropriate control arm and endpoints of clinical importance are necessary.

Acknowledgements

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HMa, SH, SI, KO, JS, HMi and NN conceptualized the study, curated the data and wrote the original draft of the manuscript. KO, RT, NO, YS, TY, YN, NS, HN, JS, HMa and NN performed the analyses, investigations and all methodology. SH, KO, RT, NO, YS, TY, YN, JS, HMi and NN performed the project administration. HMa, SH, JS and HMi wrote, reviewed and edited the manuscript. HMi and NN supervised the investigations. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Yamaguchi University School of Medicine. The requirement for informed consent was waived as the samples had been collected as part of previous clinical trials. The patients were given the opportunity to refuse that their samples be used in the present study.

Patient consent for publication

Not applicable.

Competing interests

JS has received honoraria from Tsumura, Chugai Pharmaceutical and consulting fees from Takeda Pharmaceutical. KO has received honoraria (lecture and/or manuscript fees) from Takeda Pharmaceutical Company Ltd., Bristol-Myers Squibb Company Ltd., Ono Pharmaceutical Co. Ltd. and Chugai Pharmaceutical Co. Ltd. SH and HN received research funding from NEC Corporation, Toyo Kohan Corporation and Merck Serono Co., Ltd. HMis has received honoraria from Chugai Pharmaceutical, Takeda Pharmaceutical and Taiho Pharmaceutical. NN has received honoraria from Takeda Pharmaceutical Company Ltd. The other co-authors declare that they have no competing interests.

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