DNA methylation status as a biomarker of anti-epidermal growth factor receptor treatment for metastatic colorectal cancer

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Anti-epidermal growth factor receptor (EGFR) treatment is an effective option for metastatic colorectal cancer (CRC) treatment. However, there are few reliable biomarkers to predict the clinical response to anti-EGFR treatment. We investigated the genome-wide DNA methylation status in metastatic colorectal cancer to identify associations between the methylation status and clinical response to anti-EGFR antibody. We retrospectively reviewed the medical records of 97 patients (45 patients for the first cohort and 52 patients for the second cohort) who received anti-EGFR treatment for *KRAS* wild-type metastatic CRC. Then we analyzed the associations between genome-wide DNA methylation status and clinical response to anti-EGFR treatment, and evaluated the predictive power and value of the methylation status statistically. As a result, each cohort was classified into highly methylated CRC and low methylated CRC subgroups by unsupervised clustering analyses. In the first cohort, clinical outcomes were significantly better in the low methylated CRC subgroup than in the highly methylated CRC subgroup (response rate, 35.7% *vs* 6.3%, *P* = 0.03; disease control rate, 75% *vs* 31.3%, *P* = 0.005; hazard ratio for progression-free survival, 0.27; 95% confidence interval, 0.13–0.57, *P* < 0.001; overall survival, 0.19; 95% confidence interval, 0.06–0.54, *P* < 0.001). These results were reproducible in the second cohort. The genome-wide methylation status was a predictive factor of progression-free survival and overall survival independently of *RAS* mutation status. In conclusion, we found that the genome-wide DNA methylation status is a powerful epigenetic predictor of anti-EGFR treatment in patients with *KRAS* wild-type metastatic colorectal cancer (UMIN000005490).

Colorectal cancer (CRC) is the third most common cancer and a major cause of cancer mortality worldwide.[1] Metastatic disease develops in approximately 40% of newly diagnosed patients.[2] Current standard treatment for metastatic disease is chemotherapy including molecular-targeting drugs. Standard first- and second-line treatments include fluorouracil with leucovorin and irinotecan[3,4] or oxaliplatin,[5,6] alone or in combination with bevacizumab.[7,8]

Anti-epidermal growth factor receptor (EGFR) treatment using mAb (cetuximab, panitumumab) against EGFR is effective in combination with irinotecan or as a single agent for metastatic CRC that progresses despite standard first- and second-line treatments.[9,10] Recent studies have also shown that anti-EGFR treatment is an effective option when added to irinotecan- or oxaliplatin-based therapy as the first-line treatment.[11]

Retrospective studies have shown that mutations in genes encoding key molecules within intracellular EGFR signaling pathways are associated with the inefficacy of anti-EGFR treatment.[12,13] Particularly, mutations in the *KRAS* gene at codons 12 and 13 are the most common mutations in these pathways; thus, examination of *KRAS* mutations is clinically useful to exclude patients who would be unresponsive to anti-EGFR treatment.[14,15] However, there is no other reliable biomarker to predict the clinical response to anti-EGFR treatment.

The CpG island methylator phenotype (CIMP) is a major molecular mechanism of carcinogenesis in CRC[16] and is associated with disease development in approximately 20% of cases.[17] CpG island methylator phenotype-positive CRC is reportedly associated with a higher ratio of tumors with the *BRAF* mutation and disease onset in women, the elderly, and patients with a history of smoking.[18–20] In addition to epidemiological characteristics, CIMP-positive CRC is associated with hyperplastic polyps and sessile-serrated polyps as precursor lesions.[21] Moreover, some other classification methods about the methylation status have been reported.[22] Based on these findings, prognosis and sensitivity to chemotherapy may vary according to the methylation status. Although the role of the methylation status as a prognostic factor in CRC has been

reported,[22,23] no study has examined the predictive value of the genome-wide methylation status regarding the response to CRC treatment, particularly anti-EGFR treatment.

In this retrospective study, we investigated the genome-wide DNA methylation status in metastatic CRC without *KRAS* codon 12 or 13 mutations to identify associations between methylation status and clinical response to anti-EGFR antibody.

Materials and Methods

Patients. We retrospectively reviewed the medical records of 97 patients with histologically confirmed adenocarcinoma of metastatic CRC without *KRAS* codon 12 or 13 mutation who received standard chemotherapy, including anti-EGFR antibody, at Tohoku University Hospital (TUH) (Sendai, Japan) or National Cancer Center Hospital (NCCH) (Tokyo, Japan) from 2005 to 2013. The protocol was approved by independent ethics committees of Tohoku University School of Medicine and NCCH, and all patients provided written and oral informed consent.

Tissue samples and macrodissection. Formalin-fixed paraffin-embedded (FFPE) archival tissue blocks of 97 primary tumors were obtained from patients. Unstained 10-μm-thick slices were dissected to enrich either cancer cells or normal colon mucosa under the guidance of an HE-stained tissue slide.

Extraction of genomic DNA and quality control. Genomic DNA was extracted from macrodissected samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Quality control of extracted genomic DNA was carried out by quantitative real-time PCR using the Infinium HD FFPE QC Kit (Illumina, San Diego, CA, USA) and calculated delta Cq value.

Mutation analyses. Mutations in *KRAS* exon 2 (codons 12 and 13) and *BRAF* V600E were analyzed by direct DNA sequencing (Tables S1 and S2). Infrequent-*RAS* mutations including *KRAS* (codons 61 and 146) and *NRAS* (codons 12, 13, and 61) were analyzed by Luminex Assay (GENO-SEARCH Mu-PACK, MBL, Nagoya, Japan).

Genome-wide DNA methylation analysis. Genome-wide DNA methylation analysis was carried out using the Infinium HumanMethylation450 BeadChip (Illumina) with probes that target 485 577 CpG sites and cover 99% of the RefSeq gene. The BeadChip was scanned using the iScan, and the methylation level was calculated as (β value: calculated as intensity of methylated probe/[intensity of methylated probe + intensity of unmethylated probe]). After excluding probes specific for the X and Y chromosomes, those with ≥0.25 SD of the DNA methylation β-value across all CRC samples were selected for further analyses.

Evaluation of methylation status using previously defined markers. The methylation status was also evaluated using two distinct sets of methylation markers, as reported previously. Then we evaluated whether the methylation status by each method was associated with efficacy of anti-EGFR treatment. These sets of markers were mapped on the promoter regions of: (i) seven genes (*CACANA1G, LOX, SLC30A10, ELMO1, HAND1, THBD*, and *FBN2*) reported by Yagi *et al.*[22]; and (ii) five genes (*CACNA1G, IGF2, NEUROG1, RUNX3,* and *SOCS1*) reported by Weisenberger *et al.*[18] According to the original judging systems, samples were classified into the following categories: high-methylation epigenotype (HME), intermediate-methylation epigenotype (IME), and low-methylation epigenotype (LME) by

Yagi's method, and CIMP-positive and CIMP-negative by Weisenberger's method.

Tumor response. The tumor reduction rate from treatment initiation to disease progress was evaluated in TUH and NCCH based on Response Evaluation Criteria in Solid Tumors (version 1.0) by medical oncologists. The percentage of the overall number of cases achieving a complete response or partial response or the sum of complete response, partial response, and stable disease cases was defined as the observed response rate (RR) or disease control rate (DCR), respectively.

Progression-free survival and overall survival. Progression-free survival (PFS) and overall survival (OS) were defined as the time from anti-EGFR antibody treatment to disease progression or death, respectively. Disease progression was evaluated by medical oncologists at TUH or NCCH.

Statistical analyses. Statistical analyses of patient demographic factors were carried out using the χ^2-test. Analyses of patient background factors and results of anti-EGFR treatment were carried out using JMP Pro11 software (SAS Institute Japan Co., Ltd., Tokyo, Japan). Survival curves were constructed using the Kaplan–Meier method, and differences between curves of the two subgroups were compared using the log–rank test. Associations between patient background factors, including the methylation status and PFS and OS periods after anti-EGFR antibody treatment, were used for univariate and multivariate analyses with a Cox proportional hazards model.

Probe selection in each cohort and mutual validation. We used the random forest algorithm for building the model and the sample classification. When we built the model by using one cohort dataset, the other cohort data were used as a validation test set and vice versa. First, we built two models on each cohort dataset with the common probes between the cohorts, and we selected the efficient probes. Thereafter, we constructed the models based on the fewer efficient probes, and classified the samples of the other cohort. Finally, we selected common probes from two models made in each cohort above, and classified the cohort by the model with another cohort using these common probes.

More precise methods are described in Data S1.

Results

Patients. A total of 97 primary tumor samples without *KRAS* codon 12 and 13 mutations were divided into the first cohort ($n = 45$) and second cohort ($n = 52$). The two cohorts were almost well balanced in terms of baseline characteristics (Table S3). The number of previous treatment regimens in the first cohort was significantly less than that in the second cohort ($P = 0.01$).

Genome-wide DNA methylation status. From the entire 485 577 probes, 3163 and 2577 probes within ≥0.25 SD of the DNA methylation β-value across all CRC samples were selected for the first and second cohorts, respectively. Data of the selected probes were subjected to unsupervised clustering analyses. The first cohort could be clearly classified into two subgroups, namely highly methylated colorectal cancer (HMCC, $n = 17$) and low methylated colorectal cancer (LMCC, $n = 28$) (Fig. 1a). Similarly, the second cohort was also classified into HMCC ($n = 17$) and LMCC ($n = 35$) subgroups (Fig. 1b).

Subgroup analysis of baseline characteristics according to DNA methylation status. Baseline characteristics of HMCC and LMCC subgroups were analyzed (Table 1). The primary site of the HMCC subgroup tended to be the proximal colon in

(a)

(b)
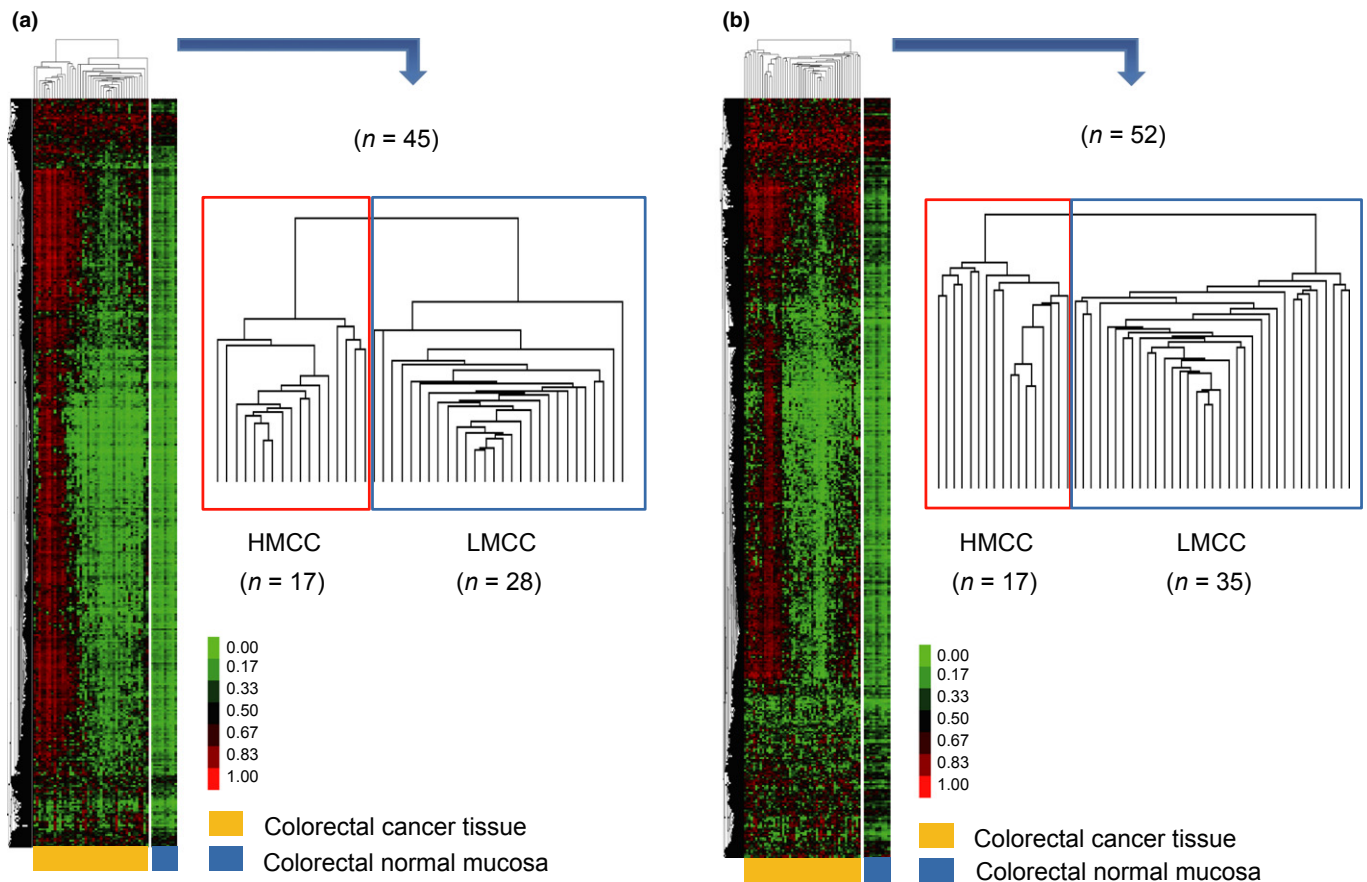
Fig. 1. Unsupervised non-hierarchical clustering of results of genome-wide DNA methylation analysis for colorectal cancer. Results of genome-wide DNA methylation analyses in the first (a) and second (b) cohorts. The first and second cohorts (n = 45 and 52, respectively) were classified into highly methylated colorectal cancer (HMCC) (n = 17 and 17, respectively) and low methylated colorectal cancer (LMCC) (n = 28 and 35, respectively) subgroups. The heat map represents the β-value (0–1.0). Each column and row represents a case and a probe, respectively.

both cohorts ($P = 0.02$ and 0.04, respectively). The number of subsequent regimens was significantly less in the HMCC subgroup than in the LMCC subgroup in the first cohort ($P < 0.01$) but not in the second cohort ($P = 0.57$). The use of the combination of irinotecan and anti-EGFR treatment was significantly less in the HMCC subgroup than in the LMCC subgroup in the first cohort ($P = 0.02$) but not in the second cohort ($P = 0.7$). There was no significant difference in other baseline characteristics between subgroups in the two cohorts.

Association between genome-wide DNA methylation status and treatment response. The association between genome-wide DNA methylation status and the clinical outcome of anti-EGFR treatment with regard to RR, DCR, PFS, and OS was analyzed. Overall RR was 25% in all cases. In the first cohort, RR and DCR were significantly higher in the LMCC subgroup than in the HMCC subgroup (35.7% *vs* 6.3%, $P = 0.03$ for RR; 75% *vs* 31.3%, $P = 0.005$ for DCR) (Table S4), and differences were validated in the second cohort (34.3% *vs* 5.9%, $P = 0.03$ for RR; 77.1% *vs* 47.1%, $P = 0.03$ for DCR). In the first cohort, hazard ratios (HRs) for PFS and OS among patients in the LMCC subgroup in comparison with the HMCC subgroup were 0.27 (95% confidence interval [CI], 0.13–0.57; $P < 0.001$) and 0.19 (95% CI, 0.06–0.54; $P < 0.001$), respectively (Fig. 2). The median periods among patients in LMCC and HMCC subgroups were 197 and 72 days for PFS, and 24.9 and 5.6 months for OS,

respectively. These results were validated in the second cohort. Hazard ratios for PFS and OS among patients in the LMCC subgroup in comparison with the HMCC subgroup were 0.22 (95% CI, 0.11–0.45; $P < 0.001$) and 0.35 (95% CI, 0.13–0.98; $P = 0.03$), respectively (Fig. 2). The median periods among patients in LMCC and HMCC subgroups were 191 and 70 days for PFS, and 14.1 and 9.3 months for OS, respectively. Furthermore, we analyzed RR, DCR, PFS, and OS in patients who used anti-EGFR as a third line of treatment. As the sample size of patients who had been administered anti-EGFR antibody for third-line treatment was small, we combined the cohorts for analysis. As a result, these findings were also confirmed only in third-line patients (Fig. S1, Table S5).

Comparison with known predictors for unresponsiveness to anti-EGFR treatment. To examine whether the differences in clinical outcome of anti-EGFR treatment between HMCC and LMCC subgroups was affected by cases with the infrequent-*RAS* mutation, 97 cases were classified into three subgroups, *RAS* wild-type HMCC subgroup (28 cases), *RAS* wild-type LMCC subgroup (58 cases), and infrequent-*RAS* mutation subgroup (11 cases), based on the results of the infrequent-*RAS* mutation analyses. The anti-EGFR treatment outcomes were then compared among these subgroups. The RR and DCR of the *RAS* wild-type HMCC subgroup were found to be significantly lower than those of the *RAS* wild-type LMCC subgroup

Table 1. Baseline characteristics of study patients of highly methylated colorectal cancer (HMCC) and low methylated colorectal cancer (LMCC) subgroups in two independent cohorts

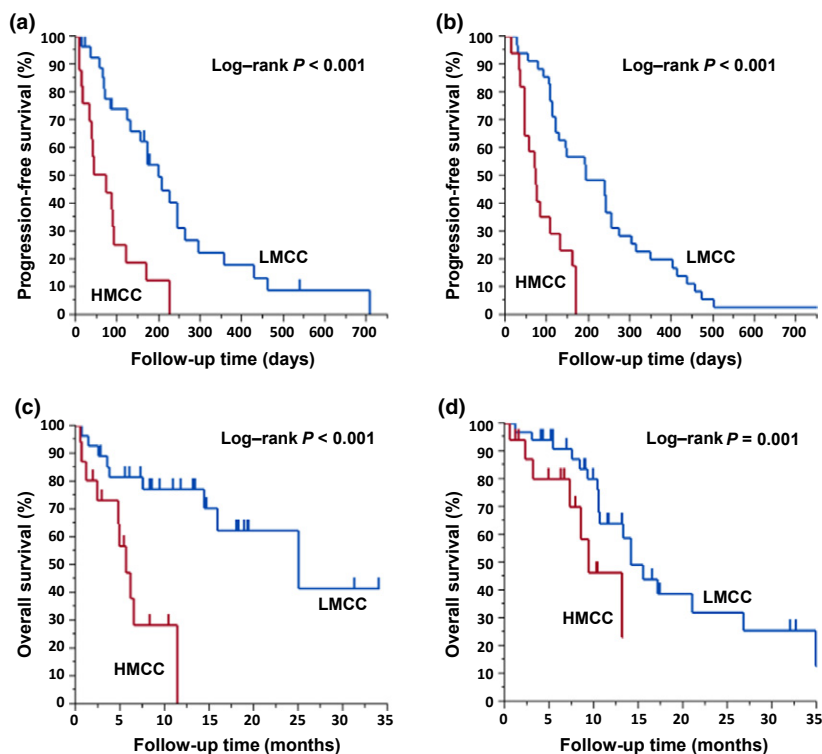| | First cohort | | | | | | | Second cohort | | | | | | |
| | All samples | | HMCC | | LMCC | | P-value | All samples | | HMCC | | LMCC | | P-value |
Variable	n	%	n	%	n	%		n	%	n	%	n	%	
Total	45	100	17	37.8	28	62.2		52	100	17	43.8	35	56.3	
Gender							0.79†							0.29†
Male	28	66.2	11	64.7	17	60.7		38	73.1	14	82.4	24	68.6	
Female	17	37.8	6	35.3	11	39.3		14	26.9	3	17.6	11	31.4	
Median age, years (range)							0.70‡							0.15‡
	61 (32–77)		61 (33–73)		61.5 (32–77)			61 (29–83)		63 (39–83)		60 (29–79)		
Primary site							0.02†							0.04†
Proximal	12	26.7	8	47.1	4	14.3		15	33.3	8	47.1	7	20.0	
Cecum	2	4.4	1	5.9	1	3.6		2	4.4	1	5.9	1	2.9	
Ascending	7	15.6	5	29.4	2	7.1		8	17.8	6	35.3	2	5.7	
Transverse	3	6.7	2	11.8	1	3.6		5	11.1	1	5.9	4	11.4	
Distal	33	73.3	9	52.9	24	85.7		37	82.2	9	52.9	28	80.0	
Descending	1	2.2	0	0.0	1	3.6		1	2.2	1	5.9	0	0.0	
Sigmoid	6	13.3	1	5.9	5	17.9		13	28.9	2	11.8	11	31.4	
Rectum	26	57.8	8	47.1	18	64.3		23	51.1	6	35.3	17	48.6	
Stage at diagnosis							0.68†							0.94†
I	0	0.0	0	0.0	0	0.0		0	0.0	0	0.0	0	0.0	
II	2	4.4	1	5.9	1	3.6		3	6.7	1	5.9	2	5.7	
III	13	28.9	6	35.3	7	25.0		17	37.8	5	29.4	12	34.3	
IV	30	66.7	10	58.8	20	71.4		32	71.1	11	64.7	21	60	
Number of organs with metastasis							0.53‡							0.84‡
0	0	0.0	0	0.0	0	0.0		2	4.4	0	0	2	5.7	
1	22	48.9	9	20	13	28.9		22	48.9	8	47.1	14	40.0	
2	21	46.7	8	17.8	13	28.9		23	51.1	7	41.2	16	45.7	
3	2	4.4	0	0.0	2	4.4		5	11.1	2	11.8	3	8.6	
BRAF mutation							0.38†							0.17†
+	3	7.5	2	11.8	1	4.3		3	5.9	2	12.5	1	2.9	
−	37	92.5	15	88.2	22	95.7		48	94.1	14	87.5	34	97.1	
NA	5		0		5			1		1		0		
No. of previous regimens							0.33‡							0.57‡
0	5	11.1	0	0.0	5	17.9		0	0.0	0	0.0	0	0.0	
1	4	8.9	2	11.8	2	7.1		5	8.2	2	11.8	3	8.6	
2	33	73.3	14	82.4	19	67.9		33	65.3	11	64.7	22	62.9	
≥3	3	6.7	1	5.9	2	7.1		14	26.5	4	23.5	10	28.6	
No. of following regimens							<0.01‡							0.57‡
0	29	64.4	17	100.0	12	42.9		33	61.2	12	70.6	21	60	
1	10	22.2	0	0.0	10	35.7		14	26.5	3	17.6	11	31.4	
≥2	6	13.3	0	0.0	6	21.4		5	10.2	2	11.8	3	8.6	
Type of anti-EGFR therapy							0.02†							0.70†
Monotherapy	12	26.7	8	47.1	4	14.3		14	26.5	4	23.5	10	28.6	
Combination with irinotecan	33	73.3	9	52.9	24	85.7		38	73.5	13	76.5	25	71.4	

†χ^2-test. ‡Wilcoxon test. NA, Not Available.

(3.7% vs 37.9%, P < 0.01 for RR; 40.7% vs 79.3%, P < 0.001 for DCR) (Table S6). The PFS and OS of the RAS wild-type HMCC subgroup were significantly shorter than that of the RAS wild-type LMCC subgroup (HR = 0.22; 95% CI, 0.13–0.37; P < 0.001; and HR = 0.24; 95% CI, 0.11–0.53; P < 0.001, respectively) (Fig. 3). The treatment outcome of the infrequent-RAS mutation subgroup was significantly inferior to that of the RAS wild-type LMCC subgroup in DCR, PFS, and OS except for RR. However, there was no significant difference in the treatment outcome of the infrequent-RAS mutation subgroup with the RAS wild-type HMCC subgroup.

A waterfall plot showing the maximal reduction rate of each case is shown in Figure 4. Among 80 RAS wild-type cases, whose reduction rates were able to be determined, 73.7% (42/57) of the RAS wild-type LMCC cases showed reduction of tumor size, whereas 78.3% (18/23) of the RAS wild-type HMCC cases showed increases in size.

Predictive power of DNA methylation status for PFS and OS. To identify factors associated with differences in PFS and OS, six parameters (methylation status, infrequent-RAS mutation status, primary site, number of previous regimens, type of anti-EGFR treatment, and BRAF mutation status) were evaluated using Cox regression analyses. In both univariate and multivariate analyses, the methylation status was the strongest predictive factor of PFS (HR = 0.23; 95% CI, 0.14–0.38; P < 0.001, HR = 0.23; 95% CI, 0.13–0.41; P < 0.001, respec-

Fig. 2. Kaplan–Meier curves for progression-free survival and overall survival after anti-epidermal growth factor receptor (EGFR) treatment in the first and second cohorts of colorectal cancer patients according to genome-wide DNA methylation status. Panels (a,b) Progression-free survival after anti-EGFR treatment for the highly methylated colorectal cancer (HMCC) subgroup (red line) and the low methylated colorectal cancer (LMCC) subgroup (blue line) in the first and second cohorts, respectively. (c,d) Overall survival after anti-EGFR treatment for the HMCC subgroup (red line) and the LMCC subgroup (blue line) in the first and second cohorts, respectively.
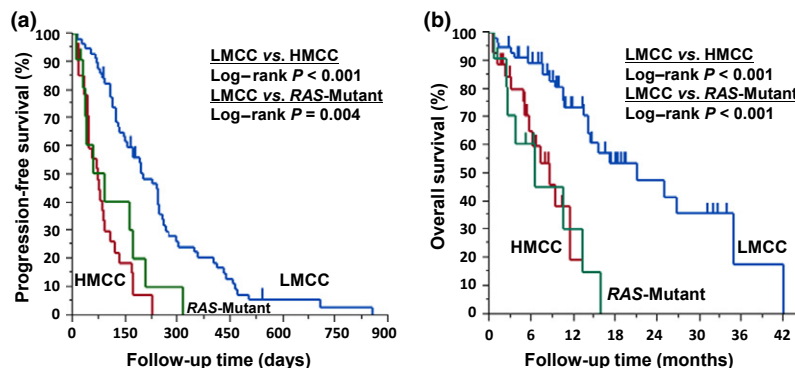
Fig. 3. Kaplan–Meier curves for progression-free survival and overall survival after anti-epidermal growth factor receptor treatment in the unified cohort (first and second cohorts) of colorectal cancer patients according to genome-wide DNA methylation status and infrequent-*RAS* mutation status. Progression-free survival (a) and overall survival (b) in the unified cohort according to *RAS*-wild highly methylated colorectal cancer (HMCC) subgroup (red line, *n* = 28), *RAS*-wild low methylated colorectal cancer (LMCC) subgroup (blue line, *n* = 58), and *RAS*-mutant subgroup (green line, *n* = 11) by Luminex Assay. (a) $\frac{\text{LMCC versus HMCC}}{\text{Log-rank } P < 0.001}$, $\frac{\text{LMCC versus RAS-Mutant}}{\text{Log-rank } P < 0.004}$. (b) $\frac{\text{LMCC versus HMCC}}{\text{Log-rank } P < 0.001}$, $\frac{\text{LMCC versus RAS-Mutant}}{\text{Log-rank } P < 0.001}$.

tively, Table 2) and OS (HR = 0.25; 95% CI, 0.12–0.50; $P < 0.001$, HR = 0.35; 95% CI, 0.16–0.74; $P = 0.006$, respectively, Table 2).

Predictive value of DNA methylation status. We compared results of classification by our method with those of classification by methylation markers used in two previous studies. First, 97 cases were divided into three subgroups according to the markers proposed by Yagi *et al.* The number of HME, IME, and LME cases was 7, 16, and 74, respectively. All HME and IME cases were classified as HMCC by our method. We then classified 97 cases by the markers reported by Weisenberger *et al.* As a result, 18 cases were considered CIMP-positive and 79 CIMP-negative. Except for one, all

CIMP-positive cases were included in the HMCC subgroup (Fig. S2).

To clarify the correlation between classification by these markers and the clinical outcome of anti-EFGR treatment, we compared clinical outcomes between subgroups classified by the reported methylation markers. As a result, there was clear correlation between methylation status and the clinical outcome of anti-EGFR treatment (Fig. S3), and our method showed the strongest correlation with the clinical outcome.

Selection of most reliable markers. To clarify the reproducibility of the classification of each cohort, we examined the prediction accuracy of the model made in each cohort
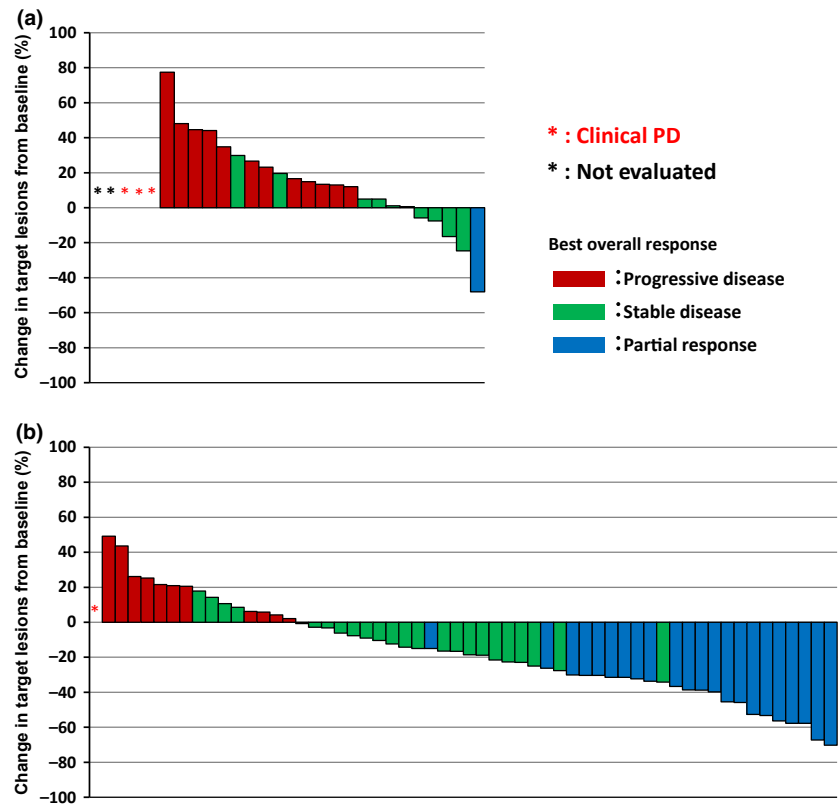
Fig. 4. Waterfall plot of the maximal reduction rate in colorectal cancer target lesions for each case. Waterfall plots include 28 highly methylated colorectal cancer (HMCC) cases without *RAS* mutations (a) and 58 low methylated colorectal cancer (LMCC) cases without *RAS* mutations (b). Each column represents a case, and the color indicates best overall response for anti-epidermal growth factor receptor treatment. Blue, partial response; green, stable disease; red, progressive disease (PD).

Table 2. Cox regression analysis for clinical outcome of anti-epidermal growth factor receptor (EGFR) therapy for colorectal cancer

| | Univariate | | | | Multivariate | | | |
| | PFS | | OS | | PFS | | OS | |
Variable	HR (95% CI)	*P*-value†	HR (95% CI)	*P*-value†	HR (95% CI)	*P*-value†	HR (95% CI)	*P*-value†
Methylation status (LMCC versus HMCC)	0.23 (0.14–0.38)	<0.001	0.25 (0.12–0.50)	<0.001	0.23 (0.13–0.41)	<0.001	0.35 (0.16–0.74)	0.006
Infrequent-*RAS* mutation status (wild versus mutant)	0.54 (0.29–1.13)	0.100	0.32 (0.15–0.76)	0.010	0.84 (0.44–1.80)	0.64	0.37 (0.17–0.91)	0.030
Primary site (distal versus proximal)	0.83 (0.53–1.34)	0.430	0.55 (0.30–1.08)	0.080	1.08 (0.65–1.83)	0.78	0.64 (0.33–1.31)	0.210
No. of previous regimens (one or less versus two or more)	0.73 (0.38–1.29)	0.290	0.44 (0.13–1.10)	0.080	0.85 (0.43–1.54)	0.61	0.48 (0.14–1.26)	0.150
Type of anti-EGFR treatment (combination with irinotecan versus monotherapy)	0.53 (0.34–0.87)	0.010	0.64 (0.31–1.45)	0.270	0.57 (0.35–0.96)	0.03	0.59 (0.28–1.39)	0.220
BRAF mutation status (wild versus mutant)	0.96 (0.42–2.82)	0.940	0.94 (0.28–5.80)	0.930	0.97 (0.41–2.89)	0.95	0.88 (0.23–5.51)	0.870

†χ^2 test. CI, confidence interval; HMCC, highly methylated colorectal cancer; HR, hazard ratio; LMCC, low methylated colorectal cancer; OS, overall survival; PFS, progression-free survival.

(see Materials and Methods) when we classified the cohort by a model made in the other cohort. We obtained 1744 common probes out of 3163 and 2577 probes, which were used in each cohort. Then we extracted the efficient 140 and 128 probes in the first cohort and second cohort, respectively, and classified the second cohort and the first cohort using either of these probes. The correct prediction rates were 94.2% (three HMCC cases were judged incorrectly) and 97.8% (one LMCC case was judged incorrectly). To further select markers that were highly reliable for prediction of anti-EGFR treatment sensitivity, we used the common 24 probes among the 140 and 128 probes (Table S7). By the model made in the first and second cohorts using the 24 probes, the correct answer rates were 98.1% (only

one HMCC case was judged incorrectly) and 100%, respectively.

Discussion

We found that metastatic CRC without *KRAS* codon 12 or 13 mutations could be clearly classified into two subgroups by genome-wide DNA methylation analysis: one-third to the HMCC subgroup and two-thirds to the LMCC subgroup. The percentage of HMCC cases was higher than that reported in previous studies (~25%), as the genome-wide analysis adopted in this study was able to detect highly methylated cases maximally (Fig. S2). We also found that genome-wide DNA methylation status was clearly correlated with the clinical outcome of anti-EGFR treatment and was an independent predictive factor of PFS and OS after anti-EGFR treatment. According to any of the distinct sets of methylation markers, the highly methylated subgroups showed inferior clinical outcomes. Importantly, as the method that is high in detectability of the DNA methylation status, predictability of the effect of treatment of the anti-EGFR therapy is high, strongly supporting this idea. In previous studies, RRs of these tumors with *KRAS* codon 12 or 13 mutation following monotherapy and combination therapy were 0–1.2% and 0–13%, respectively.[24–27] The median PFS of anti-EGFR monotherapy and combination therapy with irinotecan for cases with *KRAS* codon 12 or 13 mutations was 52–54 and 48-150 days, respectively.[24–27] Because 73.2% of cases in our cohort received anti-EGFR antibody with irinotecan (Table S3), it is rational that the clinical outcome of anti-EGFR treatment for the HMCC subgroup (RR, 6.1%; PFS, 70 days) was similar to that for CRC cases with a *KRAS* codon 12 or 13 mutation.

In addition to *KRAS* codon 12 and 13 mutations, mutations in several other codons of *KRAS* and *NRAS* (called the infrequent-*RAS* mutation) were recently established as unresponsiveness predictors of anti-EGFR treatment.[28,29] When the analysis was limited to *RAS* wild-type cases, clinical outcome of anti-EGFR treatment for the HMCC subgroup was significantly inferior to that for the LMCC subgroup and was similar to that for CRC cases with a *KRAS* codon 12 or 13 mutation (RR, 3.7%; PFS, 70 days). These results strongly support that genome-wide DNA methylation status is a predictive marker for anti-EGFR treatment outcome and could eliminate non-responders from cases without the *KRAS* codon 12 or 13 mutation, and also cases without the infrequent-*RAS* mutation.

Using a method able to evaluate the genome-wide DNA methylation status, patients who can expect benefits from anti-EGFR treatment can be narrowed down further. Obviously, we need a method that is both simple and highly reliable to assess the genome-wide DNA methylation status for clinical use. The

reproducibility of the classification in the two cohorts was thought to be actually high because the other cohort could be separated exactly when a model made in one cohort was used. Also, the probes that were important to classify both cohorts were thought to be comprised of probes with a similar methylation profile. From these results, the finally selected 24 probes can classify colorectal cancer into HMCC or LMCC subgroups with very high precision, and the transition to the detection system that is simpler and easier for clinical application is possible. We are now planning a prospective study and prospective–retrospective analysis to confirm our findings.

We did not investigate why susceptibility to the anti-EGFR antibody varied according to the DNA methylation status. *ALX4* had been identified as a chemosensitive methylation candidates to cetuximab regimens by *in vitro* chemosensitivity assay,[30] and six probes corresponding to *ALX4* were included in 1744 common probes out of 3163 and 2577 probes, which were used in each cohort. According to the epidemiological and pathological findings, biological features differ depending on the DNA methylation status.[31] Therefore, more precise mechanisms should be elucidated by further molecular analyses including whole exome sequencing and comprehensive expression analyses.

Recently, Garrido-Laguna *et al.*[32] reported that decitabine, a hypomethylating agent, in combination with panitumumab was effective in some patients previously treated with cetuximab. The susceptibility for the anti-EGFR antibody of HMCC patients may also improve by adding these agents. The methylation status and hypomethylating agents could change the treatment strategy of anti-EGFR therapy for metastatic CRC without *RAS* mutation.

This study is the first to indicate that genome-wide DNA methylation status is an epigenetic marker and independent predictive factor of anti-EGFR treatment outcomes in patients with metastatic CRC without the *KRAS* codon 12 or 13 mutation.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Detailed methods.

Data S2. Supplementary references.

Fig. S1. Kaplan–Meier curves for progression-free survival and overall survival after anti-epidermal growth factor receptor treatment in third-line patients in the unified cohort according to genome-wide DNA methylation status.

Fig. S2. Mutual relations of the cases classified in the highly methylated group by each method.

Fig. S3. Kaplan–Meier curves for progression-free survival after anti-epidermal growth factor receptor treatment in the unified cohort according to DNA methylation status.

Table S1. Polymerase chain reaction and sequencing primers for *KRAS* and *BRAF* mutation analyses.

Table S2. Polymerase chain reaction conditions for *KRAS* and *BRAF* mutation analyses.

Table S3. Baseline characteristics of the study patients in the overall population and two independent cohorts.

Table S4. Tumor response of highly methylated colorectal cancer (HMCC) and low methylated colorectal cancer (LMCC) subgroups in two independent cohorts.

Table S5. Tumor response of highly methylated colorectal cancer (HMCC) and low methylated colorectal cancer (LMCC) subgroups in third-line patients.

Table S6. Tumor response of highly methylated colorectal cancer (HMCC), low methylated colorectal cancer (LMCC), and *RAS*-mutant subgroups.

Table S7. Annotation list of selected 24 probes.