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ORIGINAL ARTICLE

Integrated analysis of cancer-related pathways affected by genetic and epigenetic alterations in gastric cancer

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Abstract

Background The profiles of genetic and epigenetic alterations in cancer-related pathways are considered to be useful for selection of patients likely to respond to specific drugs, including molecular-targeted and epigenetic drugs. In this study, we aimed to characterize such profiles in gastric cancers (GCs).

Methods Genetic alterations of 55 cancer-related genes were analyzed by a benchtop next-generation sequencer. DNA methylation statuses were analyzed by a bead array with 485,512 probes.

Results The WNT pathway was activated by mutations of *CTNNB1* in 2 GCs and potentially by aberrant methylation of its negative regulators, such as *DKK3*, *NKD1*, and *SFRP1*, in 49 GCs. The AKT/mTOR pathway was activated by mutations of *PIK3CA* and *PTPN11* in 4 GCs. The MAPK pathway was activated by mutations and gene amplifications of *ERBB2*, *FLT3*, and *KRAS* in 11 GCs. Cell-cycle regulation was affected by aberrant methylation of *CDKN2A* and *CHFR* in 13 GCs. Mismatch repair was affected by a mutation of *MLH1* in 1 GC and by aberrant methylation of *MLH1* in 2 GCs. The p53 pathway was inactivated by mutations of *TP53* in 19 GCs and potentially by aberrant methylation of its downstream genes in 38 GCs. Cell adhesion was affected by mutations of *CDH1* in 2 GCs.

Conclusions Genes involved in cancer-related pathways were more frequently affected by epigenetic alterations than by genetic alterations. The profiles of genetic and epigenetic alterations are expected to be useful for selection of the patients who are likely to benefit from specific drugs.

Keywords Epigenetics · DNA methylation · Genetic alterations · Gastric cancer · Cancer-related pathway

Abbreviations

GC	Gastric cancer
CGI	CpG island
PGM	Personal Genome Machine
TSS	Transcription start site
CIMP	CpG island methylator phenotype

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Introduction

Esophageal squamous cell carcinoma (ESCC) is a predominant histological type of esophageal cancer worldwide and one of the most serious malignant cancers due to its rapid development and fatal prognosis (Chung et al. 2010; Pennathur et al. 2013). The prognosis of patients with advanced ESCC is still unsatisfactory although aggressive treatment strategies, such as surgery with preoperative or postoperative chemotherapy or preoperative chemoradiotherapy (CRT), and definitive CRT (dCRT) (Allum et al. 2009; Ando et al. 2003, 2012; Blum et al. 2013; Conroy et al. 2014; Kato et al. 2011, 2013; Kelsen et al. 2007; Kleinberg and Forastiere 2007; Tepper et al. 2008; van Hagen et al. 2012), have been implemented. Among these aggressive treatments, dCRT has been an important therapeutic strategy for advanced ESCC (Conroy et al. 2014; Kato et al. 2011, 2013; Kleinberg and Forastiere 2007; van Hagen et al. 2012). By dCRT, a complete response of primary tumor is seen in 58.0–70.6 % of patients (Kato et al. 2011, 2013; Tahara et al. 2005), and such patients not only have a good prognosis but also can preserve their esophagus and keep a better quality of life than patients who undergo esophagectomy. In contrast, survival benefits of dCRT for patients who have residual tumors are limited even after adequate salvage treatments (Conroy et al. 2014; Kato et al. 2011, 2013). Although preoperative chemo- or chemoradiotherapy followed by surgery may be powerful, operative mortality and morbidity are not ignored. If we can predict the response of ESCC to dCRT before starting a treatment, we can stratify patients who will benefit by it for personalized treatments.

To establish a biomarker to predict the response of ESCC to dCRT, a lot of effort has been made in the field of molecular markers, and markers using specific RNA and protein expression have been reported (Akutsu et al. 2011; Gao et al. 2013; Makuuchi et al. 2013; Okamoto et al. 2013; Zhang et al. 2013). Although these markers were associated with the response to dCRT, their utility has not been confirmed by independent studies yet. Also, their accuracy even in the screening set was not satisfactory for clinical use. Therefore, further searching for a predictive marker for dCRT is required, and validating the utility of the marker is essential for clinical application.

As a molecular marker, DNA methylation has several advantages over RNA and proteins (Goel 2010; Issa 2012; Laird 2003). First, DNA methylation status is stable, even if a cell is placed in different environments, and consistent results can be obtained even under different conditions of sample collection where gene expression profiles are affected. Secondly, DNA methylation can be analyzed using DNA, which can remain relatively intact in samples with RNA and protein degradation. Thirdly, DNA methylation has only two statuses, methylated or unmethylated, and its profile cannot be affected by a small amount of contaminating cells, different from gene expression. Also, DNA methylation status has been reported to correlate with clinicopathological features of many types of cancers (Brait et al. 2008; Kim et al. 2013; Sato et al. 2002; Yang and Park 2012). Taking these advantages, DNA methylation markers for the prediction of a response to a cancer therapy have been identified in other types of cancers (Amatu et al. 2013; Giovannetti et al. 2012; Hegi et al. 2005; Mikeska et al. 2012; Park et al. 2009; Toyota et al. 2009). However, in ESCC, few studies have demonstrated an association between DNA methylation status and response to dCRT (Brabender et al. 2009).

In the present study, we aimed to identify genomic regions whose methylation statuses are associated with the response of ESCC to dCRT by a genome-wide methylation analysis and to validate the isolated candidate predictive markers for the response to dCRT.

Methods

Samples and patient profiles

A total of 187 ESCC samples were collected from ESCC patients from January 2006 to April 2013 at the National Cancer Center Hospital and the Osaka City University Hospital. This study was approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan (Reference No. 2010-094), and the Osaka City University, Osaka, Japan (Reference No. 1500). Written informed consents were obtained from all the individuals. They had histologically confirmed ESCC and were at cStage II–IV according to the 6th edition of the TNM classification (Sobin 2002). Patients with clinical T4 and with distant organ metastasis were excluded.

A group of 128 patients underwent chemotherapy comprised of two courses of infusion of 5-fluorouracil (5-FU) (700 mg/m^2/day) on days 1–4 and 29–32 and 2-h infusion of cisplatin (CDDP) (70 mg/m^2) on days 1 and 29. A total of 60 Gy was concurrently administered in 30 fractions. A 1-week break was provided after 30 Gy irradiation, and radiotherapy was resumed on day 29 with the second chemotherapy course. The other group of 59 patients underwent chemotherapy comprised of two courses of infusion of 5-FU (1,000 mg/m^2/day) on days 1–4 and 29–32 and a 2-h infusion of CDDP (75 mg/m^2) on day 1 and 29. A total of 50.4 Gy was concurrently administered in 28 fractions. Break period of radiation was not planned. An additional two cycles of chemotherapy were conducted after completion of the radiotherapy until the tumor disappeared or progressed.

The response to dCRT was determined based on the findings of endoscopic evaluation of the primary tumor after each course of chemotherapy using the modified criteria of the 10th edition of the Japanese Society for Esophageal Diseases (Kuwano et al. 2008). A responder was defined as a patient with disappearance of the primary tumor without the presence of irregular erosion, ulceration, visible elevated lesions, acute esophagitis, or malignant cells in biopsy specimens. A non-responder was defined as a patient not diagnosed as a responder after finishing all courses of chemotherapy.

DNA extraction, estimation of the fraction of cancer cells in a DNA sample, and sample selection

The 187 ESCC samples consisted of 48 biopsy samples stored in RNAlater (Applied Biosystems, Foster City, CA, USA) at −80 °C (frozen samples), and 139 biopsy samples embedded in paraffin wax block after fixation with formalin (FFPE samples). Genomic DNA was extracted by using the phenol/chloroform method. The fraction of cancer cells in a DNA sample was assessed using a DNA methylation marker for the fraction of cancer cells (Takahashi et al. 2013).

Among the 48 frozen samples, five samples were excluded from further analysis because the fraction of cancer cells was <20 % (four samples) or the total number of DNA molecules assessed by quantitative methylation-specific PCR (qMSP) was <50 (1 sample). As a result, the 43 remaining frozen samples were used as the screening set (Table 1). Among the 139 FFPE samples, 78 samples were excluded from further analysis because the fraction of cancer cells was <20 % (two samples) or the total number of DNA molecules was <50 (76 samples). Consequently, the 61 remaining FFPE samples were used as the validation set (Table 1).

Genome-wide DNA methylation analysis

A genome-wide screening of differentially methylated CpG sites was conducted using an Infinium HumanMethylation450 BeadChip array, which covered 482,421 CpG sites (Illumina, San Diego, CA, USA) (Shigematsu et al. 2012). We excluded 11,551 CpG sites on the sex chromosomes and used the remaining 470,870 CpG sites for the analysis. To adjust probe design biases, intra-array normalization was conducted using a peak-based correction method, Beta MIxture Quantile dilation (BMIQ) (Teschendorff et al. 2013). The methylation level of each CpG site was represented by a β value, which ranged from 0 (completely unmethylated) to 1 (completely methylated).

qMSP and normalization of the methylation levels

Sample DNA was treated with sodium bisulfite and purified (Kaneda et al. 2004). qMSP was conducted by real-time PCR using 25 ng bisulfite-modified DNA and specific primers (Supplementary Table 1), SYBR Green I (BioWhittaker Molecular Applications, Rockland, MD, USA), and an MyiQ real-time PCR detection system (Bio-Rad Laboratories, CA, USA). Standard DNA for a methylated sequence was prepared by purifying PCR products with primers for the methylated sequence and genomic DNA treated with *Sss*I methylase (New England Biolabs, Beverly, MA, USA). That for an unmethylated sequence was prepared by purifying PCR products with primers for the unmethylated sequence and DNA amplified twice with a GenomiPhi HY DNA amplification kit (GE Healthcare Bio-Science, Buckinghamshire, England). The numbers of methylated and unmethylated molecules in a sample were calculated by comparing its amplification with that of methylated and unmethylated standard DNA, respectively, that contained known numbers of molecules (10–10^6 molecules).

A methylation level was calculated as the fraction of methylated molecules in the total number of DNA molecules (the number of methylated molecules + the number of unmethylated molecules) in a sample (Oka et al. 2009). The measured methylation level was normalized for each gene using the fraction of cancer cells in a sample [the normalized methylation level = 100 × (the measured methylation level (%))/(the fraction of cancer cells in the sample (%))].

Statistical analysis

Fisher's exact test was used to evaluate the significant difference in relative frequency between two independent groups. Differences in the normalized methylation levels between the responders and the non-responders were evaluated by Mann–Whitney *U* test. In the univariate analysis, the odds ratios (ORs) and 95 % confidence interval (95 % CI) were calculated. The confounders affecting the apparent likelihood of the response to dCRT were tested using multivariate logistic regression analysis. Overall survival was calculated from the initiation of the first course of treatment to the date of death or final date of survival confirmation. Survival curves were drawn by the Kaplan–Meier method and were compared by the log-rank test. All statistical analyses were conducted by PASW statistics version 18.0.0 (SPSS Japan Inc., Tokyo, Japan).

Results

Genome-wide screening to isolate genomic regions whose methylation statuses were associated with the response to dCRT in the screening set

To isolate genomic regions specifically methylated or unmethylated in responders, a genome-wide methylation analysis was performed using an Infinium HumanMethylation450 BeadChip array. For this analysis, we used (1) seven ESCC samples from responders with 40 % or more of cancer cells, (2) eight ESCC samples from non-responders with 40 % or more of cancer cells, (3) peripheral leukocytes of one healthy volunteer, (4) a pool of normal esophageal mucosae of four healthy volunteers, and (5) a pool of non-cancerous esophageal mucosae of eight ESCC patients. The ESCC samples were selected from 43 samples in the screening set (Table 1).

From 470,870 CpG sites on autosomes, we selected 165,081 CpG sites unmethylated (β value <0.2) in the peripheral leukocytes and the pool of normal esophageal mucosae Fig. 1. From the 165,081 CpG sites, 156,381 CpG sites unmethylated (β value <0.2) in the pool of non-cancerous mucosae were selected. From the 156,381 CpG sites, we isolated 543 CpG sites (1) specifically methylated [β value $\geq$ cancer cell fraction (%)/200] in the responders and (2) by which the response to dCRT was predicted in 11 or more of the 15 ESCCs. Finally, from the 543 CpG sites,

Table 1 Clinicopathological data and the fraction of cancer cells in the screening and validation sets

Feature	Genome-wide analysis set[a]			Screening set			Validation set		
	Responder	Non-responder	*P* value[b]	Responder	Non-responder	*P* value[b]	Responder	Non-responder	*P* value[b]
Number of patients	7	8		23	20		41	20	
Age (years)[c]	66.1 ± 4.4	66.8 ± 5.9	0.827	67.4 ± 6.3	65.1 ± 8.3	0.318	66.0 ± 8.1	60.5 ± 7.7	0.014
Gender									
Male	6	5	0.569	20	15	0.44	35	19	0.258
Female	1	3		3	5		6	1	
Location									
Cervical	1	0	0.709	2	0	0.116	7	3	0.247
Upper	1	2		1	5		3	4	
Middle	4	5		17	11		14	9	
Lower	1	1		3	4		17	4	
Radiation dose (Gy)									
60	5	5	1	11	8	0.76	33	12	0.083
50.4	2	3		12	12		8	8	
cT									
1	1	1	0.529	5	2	0.016	14	0	0.001
2	1	0		6	0		7	2	
3	5	7		12	18		20	18	
cN									
0	1	2	1	3	2	1	7	2	0.377
1	6	6		20	18		34	18	
cM									
0	5	5	1	18	10	0.064	33	13	0.158
1	2	3		5	10		8	7	
cStage									
2	2	3	0.765	11	4	0.09	23	2	0.003
3	3	2		7	6		10	11	
4	2	3		5	10		8	7	
Fraction of cancer cells (%)[c]	65.6 ± 16.1	63.5 ± 14.2	0.793	59.7 ± 19.3	57.9 ± 17.6	0.756	64.1 ± 17.1	60.3 ± 17.6	0.417

[a] This set was derived from samples in the screening set

[b] *P* values were calculated by the chi-square test

[c] Mean ± SD

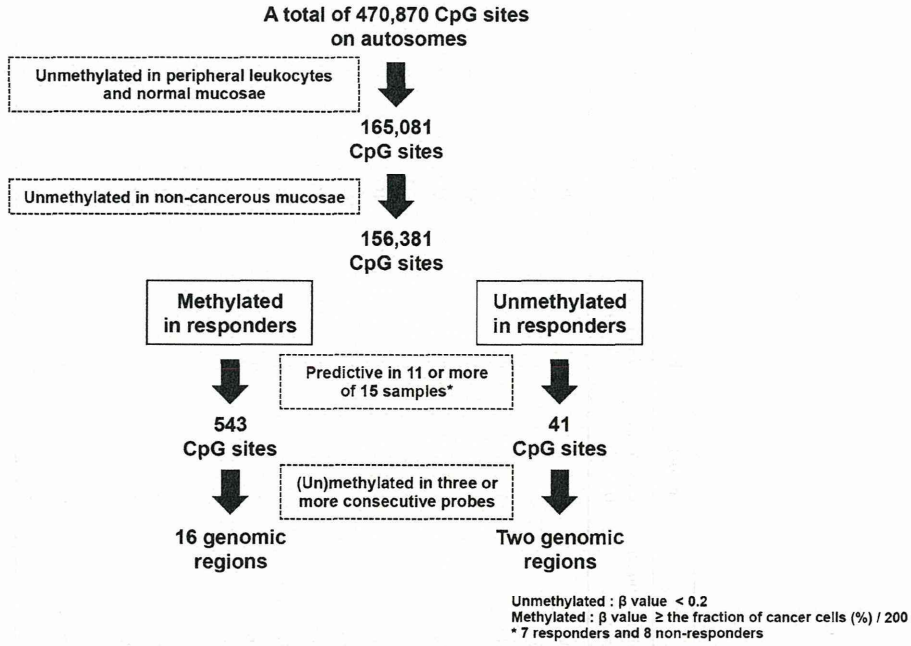

Fig. 1 Selection processes of genomic regions whose methylation statuses were associated with the response to dCRT. From the 482,421 CpG sites on the Infinium HumanMethylation450 BeadChip array, those on autosomes and unmethylated in non-cancerous mucosae were selected. As specifically methylated in responders, 543 CpG sites, resulting in 16 genomic regions, were isolated. As specifically unmethylated in responders, 41 CpG sites, resulting in two genomic regions, were isolated. An unmethylated CpG site was defined when its β value was <0.2. A methylated CpG site was defined when its β value was more than or equal to half of the fraction of cancer cells in a sample [β value $\geq$ the fraction of cancer cells in the sample (%)/200]

we isolated 16 genomic regions methylated or unmethylated in three or more consecutive probes in each sample. In the same way, we isolated two genomic regions specifically unmethylated (β value <0.2) in the responders (Table 2; a representative locus in Supplementary Fig. S1). Among the 18 genomic regions, we were able to design primers for qMSP in six regions.

Selection of genomic regions whose methylation statuses were associated with the response to dCRT in the screening set

To select genomic regions whose methylation statuses were associated with the response to dCRT, methylation levels of the six genomic regions were quantified in the screening set (Table 1) by qMSP. To exclude the effect of contamination of non-cancerous cells in a sample, a normalized methylation level was calculated using the fraction of cancer cells in a sample assessed using a DNA methylation marker (Takahashi et al. 2013) [the normalized methylation level = 100 × (the measured methylation level (%))/(the fraction of cancer cells in the sample (%))]. Among the six genomic regions, the normalized methylation levels of the promoter CpG island of *ZNF695* in the responders were significantly higher than those in the non-responders ($P = 0.004$; Fig. 2).

For *ZNF695*, a cutoff value of 8.0 was established, so that the Youden index (sensitivity + specificity − 1) would be maximized. Also, we implemented a criterion that a cutoff value should be larger than 2.0 because methylation levels in this range were experimentally reliable. Using this cutoff value, the incidence of *ZNF695* methylation was significantly higher in the responders than in the non-responders ($P = 0.004$).

Validation of the association between *ZNF695* methylation and the response to dCRT

To validate the association between *ZNF695* methylation and the response to dCRT, its methylation levels were quantified in an independent sample set (validation set, Table 1) and were normalized by the fraction of cancer cells in a sample. The normalized methylation level of *ZNF695* was significantly higher in the responders than in the non-responders ($P = 0.021$; Fig. 3). Using the cutoff value of 8.0, which had been prefixed in the screening set, the incidence of *ZNF695* methylation in the responders was significantly higher than that in the non-responders ($P = 0.036$). These results demonstrated that the association between *ZNF695* methylation and the response to dCRT was valid even in an independent sample set and that *ZNF695* methylation was a candidate predictive marker for the response to dCRT. The response to dCRT was detected with a sensitivity of 39 %, specificity of 90 %, and positive predictive value of 89 %.

Association between *ZNF695* methylation and clinicopathological characteristics

The predictive power of *ZNF695* methylation was compared with other clinical factors. Univariate analyses showed that age, clinical T stage, clinical M stage, and

Table 2 Genomic regions identified by genome-wide methylation analysis

No.	Gene symbol	Location		Position against a gene	Relation to a CpG island	Mean β value		Incidence of methylation		P value[a]	
		Chr	nt. number			Responder ($n = 7$)	Non-responder ($n = 8$)	Responder ($n = 7$)	Non-responder ($n = 8$)	Screening	Validation
1	*AGRN*	1	976168–976227	Body	Island	0.30	0.04	4	0	ND	ND
2	*ZNF695**	1	247171252–247171572	1stExon-TSS1500	Island-S_Shore	0.29	0.02	4	0	0.004	0.021
3	*HOXD8**	2	176994665–176995088	5′UTR	Island	0.33	0.10	4	1	0.108	ND
4	*TRIM71*	3	32859377–32859445	TSS200	Island	0.24	0.04	3	0	ND	ND
5	*PCDHA7**	5	140306181–140306213	Body	Island	0.32	0.11	4	1	0.289	ND
6	*KIAA1949*	6	30653659–30653736	Body	N_Shore	0.35	0.15	4	1	ND	ND
7	*LEMD2*	6	33739406–33739607	3′UTR	Island	0.31	0.08	4	0	ND	ND
8	*PCLO*	7	82792105–82792284	5′UTR-TSS200		0.24	0.06	3	0	ND	ND
9	*C12orf56**	12	64784252–64784626	1stExon-TSS1500	Island	0.40	0.16	6	2	0.053	ND
10	*MEIS2*	15	37390176–37390326	1stExon	Island	0.49	0.27	6	3	ND	ND
11	*CCDC64B*	16	3079708–3079953	Body	Island	0.28	0.06	3	0	ND	ND
12	*FAM38A*	16	88850218–88850534	Body	Island	0.36	0.10	4	0	ND	ND
13	*ZNF585B**	19	37701550–37701642	TSS200		0.25	0.01	3	0	0.357	ND
14	*ZNF155*	19	44488121–44488181	TSS200		0.27	0.03	3	0	ND	ND
15	*ZNF350*	19	52490223–52490339	TSS200-TSS1500		0.22	0.05	4	0	ND	ND
16	*GALR3**	22	38221009–38221187	Body	Island	0.37	0.11	4	1	0.154	ND
17	*CXCL5*	4	74864165–74864313	1stExon	Island	0.36	0.59	4	8	ND	ND
18	*PKD2L2*	5	137224904–137225191	TSS1500-1stExon	N_Shore-Island	0.07	0.29	1	4	ND	ND

Chr, chromosome number; nt. number, nucleotide number in the NCBI database (NCBI37/hg19); TSS200, within 200 bp upstream of the transcription start site (TSS); TSS1500, 200–1,500 bp upstream of a TSS; body, within exons or introns of a gene; 3′UTR, 3′ untranslated region of a gene, 5′UTR; 5′ untranslated region of a gene; Island, CpG island; S_Shore, within 2,000 bp from the end of a CpG island; N_Shore, within 2,000 bp of the start of a CpG island; and ND, not done

* Primers for qMSP were successfully designed

[a] Difference was evaluated by Mann–Whitney U test

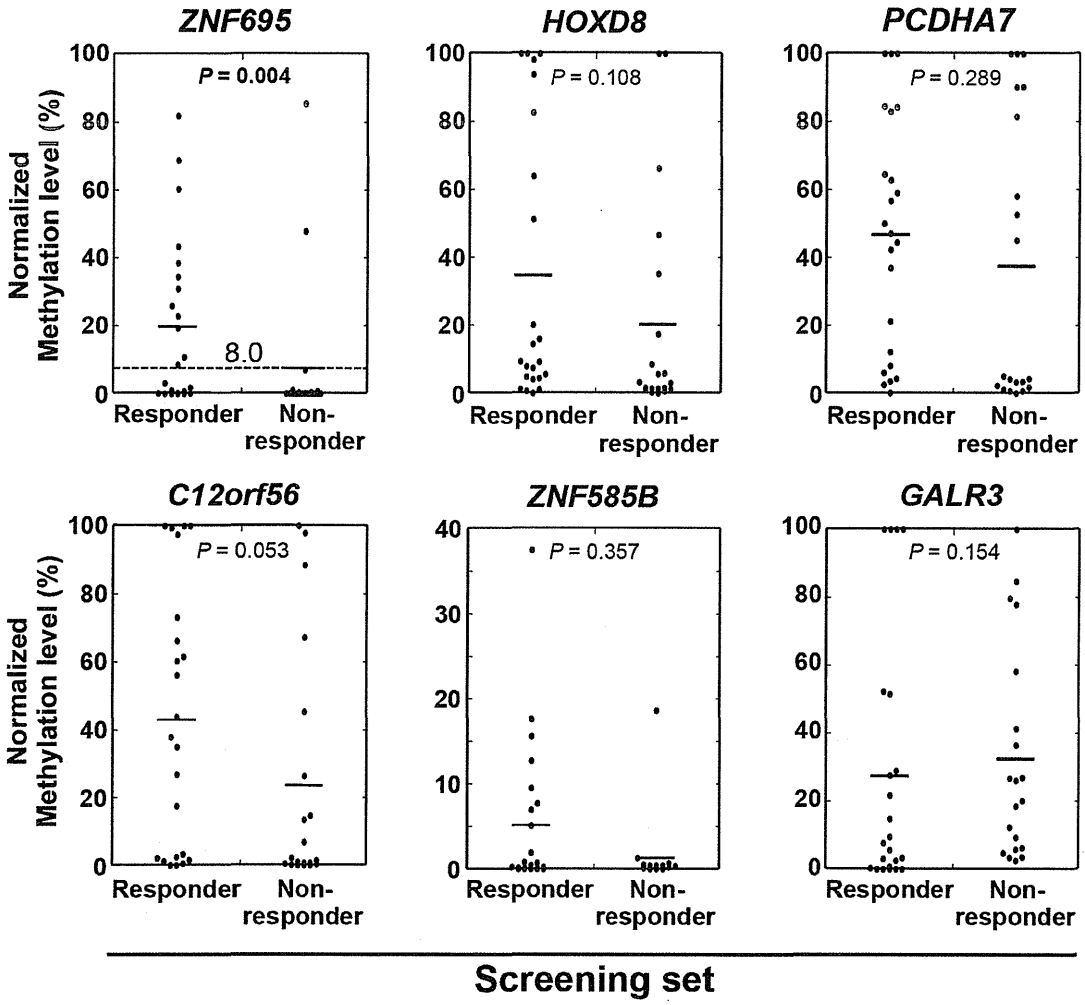

Fig. 2 Normalized methylation levels of the candidate genomic regions in the screening set. Methylation levels of six genomic regions were measured by qMSP in 23 responders and 20 non-responders in the screening set. A normalized methylation level was calculated using the fraction of cancer cells in a sample [the normalized methylation level = 100 × (the measured methylation level (%))/(the fraction of cancer cells in the sample (%))]. The *horizontal dotted line* shows a cutoff value of 8.0 adopted for *ZNF695*

ZNF695 methylation were significantly associated with the response to dCRT (Table). A multivariate logistic regression analysis using the age, sex, radiation dose, clinical T stage, clinical M stage, and *ZNF695* methylation showed that, in addition to the clinical T stage, *ZNF695* methylation was an independent predictive factor for the response to dCRT (OR 7.55, 95 % CI 2.12–26.9, $P = 0.002$; Table).

Finally, by Kaplan–Meier analysis, patients with the response to dCRT had significantly better overall survival than those without the response to dCRT ($P < 0.001$; Supplementary Fig. S2a). However, *ZNF695* methylation (methylated vs. unmethylated) and clinical T stage (cT1 or T2 vs. cT3) were not significantly associated with overall survival (methylated vs. unmethylated, $P = 0.469$; cT1 or T2 vs. cT3, $P = 0.244$; Supplementary Fig. S2b and S2c). Also, by Kaplan–Meier analysis stratified by clinical T stage, *ZNF695* methylation (methylated vs. unmethylated) was not significantly associated with overall survival (cT1 or T2, $P = 0.559$; cT3, $P = 0.593$; Supplementary Fig. S3a and S3b).

Discussion

In the present study, a candidate predictive marker for the response of ESCC to dCRT, *ZNF695* methylation, was successfully identified by a genome-wide methylation analysis. Because any genome-wide screening has a risk of obtaining "too good" results due to overfitting, a biomarker isolated by such a screening needs to be validated

using a different set of samples. Here, the predictive power of *ZNF695* methylation was confirmed in an independent set of samples ($P = 0.021$), and the concern of overfitting was resolved.

The specificity and sensitivity in the validation set were 90 and 39 %, respectively, using the cutoff value established in the screening set. The multivariate analysis showed that *ZNF695* methylation was an independent predictive factor. These results indicated that a patient with *ZNF695* methylation can achieve a complete response of a primary tumor with high probability. Since the risk of failing to respond to dCRT is the major reason why some clinicians are reluctant to recommend dCRT to patients, this characteristic of *ZNF695* methylation may become a great advantage in the selection of appropriate treatments. In contrast, even among the patients within a specific clinical tumor stage, *ZNF695* methylation was not significantly associated with the response to dCRT, due to the low sensitivity of *ZNF695* methylation. Therefore, if a patient does not have *ZNF695* methylation, we cannot conclude that the patient will not benefit by dCRT. To overcome the low sensitivity, a combination with other high specificity markers might be useful.

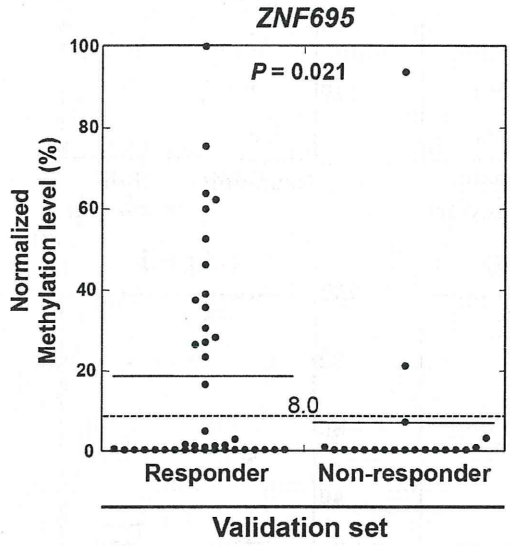

Fig. 3 Normalized methylation levels of *ZNF695* in the validation set. Methylation levels of *ZNF695* were measured by qMSP in 41 responders and 20 non-responders in the validation set. A normalized methylation level was calculated as described in the legend of Fig. 2. The *horizontal dotted line* shows the cutoff value of 8.0 prefixed in the screening set

In general, patients with a complete disappearance of a primary tumor have better prognosis that those with a residual primary tumor (Tahara et al. 2005). Also in this study, the responders showed significantly better survival than the non-responders (Supplementary Fig. S2a). It is therefore expected that a biomarker to predict the response to dCRT could also predict the prognosis of patients. However, *ZNF695* methylation was not associated with better overall survival. Among the patients with unmethylated *ZNF695*, more than half of the patients were responders due to the low sensitivity of this marker. This was considered as a reason why we were not able to observe a significant difference in overall survival between patients with methylated and unmethylated *ZNF695*.

Table 3 Univariate analysis of the response to dCRT

Features	Categories	No. of cases	Responder	Non-responder	OR	95 % CI	*P* value[a]
Age	≥60	83	57	26	4.84	1.58–12.2	0.005
	<60	21	7	14			
Gender	Female	15	9	6	1.08	0.35–3.30	1.000
	Male	89	55	34			
Location	Cervical, upper	25	13	12	1.68	0.68–4.18	0.346
	Middle, lower	79	51	28			
Clinical T stage	T1, T2	36	32	4	9.00	2.87–28.2	<0.001
	T3	68	32	36			
Clinical N stage	N0	14	10	4	1.67	0.49–5.74	0.558
	N1	90	54	36			
Clinical M stage	M0	64	51	23	2.90	1.21–6.95	0.025
	M1	40	13	17			
Radiation dose (Gy)	60	69	45	24	1.58	0.69–3.62	0.294
	50.4	35	19	16			
ZNF695 methylation	Methylated	32	28	4	7.00	2.23–22.0	<0.001
	Unmethylated	72	36	36			

[a] *P* values were calculated by Fisher's exact test

Table 4 Multivariate logistic regression analysis to identify independent factors

	No. of cases	OR	95 % CI	*P* value
Clinical T category (cT1 or cT2 vs. cT3)	36/68	7.58	2.07–27.8	0.002
ZNF695 methylation (methylated vs. unmethylated)	32/72	7.55	2.12–26.9	0.002
Age (≥60 vs. <60)	83/21	2.25	0.66–7.68	0.196
Clinical M category (cM0 vs. cM1)	64/40	2.18	0.75–6.32	0.153
Radiation dose (60 vs. 50.4)	69/35	1.96	0.68–5.61	0.213
Gender (male vs. female)	89/15	0.73	0.19–2.82	0.647

One advantage of DNA methylation marker is that DNA methylation can be assessed using DNA, which is chemically stable. In our study, we used samples fixed with formalin and embedded in paraffin wax. Even using the samples with which analysis of RNA and most proteins is difficult, we were able to assess a methylation level of *ZNF695* and to use the samples for the analysis. However, we had to exclude samples in which the total number of DNA molecules assessed by qMSP was <50. This resulted in the selection of 61 FFPE samples from the initial 139 FFPE samples. It is known that storage in formalin for a prolonged period results in the degradation of DNA (Bonin et al. 2010; Funabashi et al. 2012; Turashvili et al. 2012), and use of samples with shorter storage is considered desirable.

Contamination of non-cancerous cells in a tumor DNA sample can affect the results of any DNA methylation analysis (Loh et al. 2010). To overcome this issue, we excluded samples with low cancer cell content (<20 %) from the analysis and normalized the measured methylation level by the fraction of cancer cells in a sample assessed by a DNA methylation marker (Takahashi et al. 2013). The normalized methylation level was considered to well reflect the methylation status of cancer cells in a sample, compared with the actual methylation level measured. By comparison of the normalized methylation levels between the responders and non-responders, we were able to analyze subtle difference in methylation status between the two groups.

The biological basis of why *ZNF695* methylation was associated with the response to dCRT needs to be established. The methylated genomic region was located in a CpG island in the promoter region, and its methylation is considered to be critical for the regulation of gene expression. Because *ZNF695* is a zinc finger protein and likely to be a transcription factor, one possible mechanism is that *ZNF695* protein regulates the expression of genes involved in DNA repair introduced by therapy, including radiation, cisplatin, or 5-FU. Another possible mechanism is that a mechanism that alters methylation of multiple genes, such as the CpG island methylator phenotype, causes *ZNF695* methylation, and the mechanism is critical for cancer cells surviving dCRT. Recently, splice variants of *ZNF695* have been reported to be highly expressed in ovarian cancer (Juarez-Mendez et al. 2013). Although it is still unknown whether such splice variants are associated with aggressive behavior of ovarian cancer, there is a possibility that by loss of *ZNF695* function, cancer cells acquire a high proliferation ability, resulting in resistance to therapy.

In conclusion, we identified that methylation of the promoter CpG island of *ZNF695* was associated with the response of ESCC to dCRT with a high specificity of 90 %. *ZNF695* methylation is a promising predictive marker for dCRT, and a large-scale analysis is warranted.

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Conflict of interest None.

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