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Simple Cancer Stem Cell Markers Predict Neoadjuvant Chemotherapy Resistance of Esophageal Squamous Cell Carcinoma

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Abstract. *Background/Aim: Cancer stem cells (CSCs) contribute to resistance against neoadjuvant chemotherapy (NAC) in esophageal squamous cell carcinoma (ESCC). We conducted a retrospective observational study for the relationship between the expression levels of CSC markers in biopsy specimens prior to 5-fluorouracil plus cisplatin (FP)-NAC and the pathological responses. Patients and Methods: We included 171 patients with ESCC who underwent the FP-NAC followed by radical resection. Biopsy specimens prior to the FP-NAC were obtained and immunochemically stained for CD44, CD133, and CD24. Results: The biopsy specimens of the non-responders had the $CD44^{high}/CD24^{low}$ expression at high levels, which was found as an independent predictor of not only FP-NAC resistance but also poor overall survival by multivariate analyses. Conclusion: $CD44^{high}/CD24^{low}$ expression in the biopsy specimens prior to FP-NAC may be a predictor of FP-NAC resistance and poor prognosis of ESCC patients.*

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Esophageal cancer is the sixth most deadly cancer worldwide. It comprises two main histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC occurs mainly in East Asia, including Japan, whereas EAC occurs mainly in Western countries (1). For patients with ESCC, neoadjuvant therapies (NATs) followed by radical resection have become the standard treatment because this strategy has been shown to improve the overall survival (OS) and resection rates compared with surgery alone (2, 3). Among the NATs, neoadjuvant chemotherapy (NAC) combined with 5-fluorouracil and cisplatin (FP) has been shown to promote better therapeutic outcome by the Japan Clinical Oncology Group trial #9907 and become a standard preoperative treatment. However, even if the ESCC patients undergo NAC with FP (FP-NAC) followed by radical resection the prognosis remains poor with a 5-year survival rate of 54.8% (4, 5).

In general, NATs include a process to evaluate therapeutic responses to the NATs themselves in the surgical specimens, which provides a unique opportunity to identify potent predictors of NAT responders (6). In the cases of ESCC treated with the FP-NAC, the pathological responses of surgical specimens to the FP-NAC have been shown to be

associated with prognosis. Therefore, identifying predictors of the NAT responders may contribute to personalize treatment options and avoid toxicity (4). Currently, however, there are no appropriate predictors for ESCC that precisely predict the pathological responses to NATs.

Cancer stem cells (CSCs) are responsible for the initiation, progression, metastasis, and therapy resistance of cancers. CSCs are characterized by the expression of cell surface markers, such as CD44, CD133, CD24, and LGR5 (7, 8). Therefore, CSC markers are potentially useful to predict the therapeutic responses. In this regard, we have previously analyzed the CSCs that were flow-cytometrically isolated from surgically resected cancer tissues (9). However, as this method was time-consuming and complicated in the tissue processing stage, we found it not suitable as a screening method to predict those who would benefit from NATs. In this study, we aimed at determining practical and clinically feasible methods to predict the pathological responses to the NATs in ESCC. To this end, we evaluated biopsy specimens prior to FP-NAC and immunohistochemically stained them with CD44, CD133, and CD24. Among these markers, CD44 is the most frequently used marker to identify cells with CSC characteristics in various tumors, including ESCC (6, 7). However, ESCCs have been shown to be positive for CD44 at too high levels (10, 11), which suggest that CD44 alone would be insufficient to specify ESCC-CSCs precisely. Therefore, better combinations with other markers are required. From this viewpoint, CD133 and CD24 have been considered as candidate markers of the ESCC-CSCs for the following reasons. The positive expression of CD133 in combination with CD44 ($CD44^+CD133^+$) has been shown to be a valuable marker of the ESCC-CSCs (12, 13). Besides, as the co-expression of CD44 at high levels and CD24 at low levels ($CD44^+CD24^{-/low}$) has initially been considered as a CSC marker of breast cancers (14), $CD44^+CD24^-$ has been reported as a therapy-resistant marker of the ESCC-CSCs (15, 16).

Based on these findings, we hypothesized that certain combinations of the known CSC markers expressed in the biopsy specimens of ESCC prior to FP-NAC would be associated with the pathological responses in the surgical specimens or the prognosis of the ESCC patients. To this end, we conducted a retrospective observational study to analyze the relationship between the expression levels of the CSC markers in the biopsy specimens prior to FP-NAC by immunohistochemistry, the pathological responses in the surgical specimens, and the prognosis of the ESCC patients.

Patients and Methods

Patients. This retrospective study was conducted with the approval of the Institutional Review Board and the Ethics Committee of the Graduate School of Medicine, Kobe University School of Medicine (approval number B190065). We included the patients with ESCC who underwent FP-NAC followed by radical esophagectomy at Kobe University Hospital from January 2008 to December 2016 and met the following criteria: 1) ESCC was confirmed histologically and by biopsy prior to the FP-NAC, 2) two cycles of the FP chemotherapy were completed, 3) R0/R1 resection was performed, and 4) biopsy specimens prior to the FP-NAC were available. Sections were histologically analyzed according to the Union for International Cancer Control tumor, node, metastasis (TNM) classification (7^{th} edition) system. Evaluation of patients' survival was based on the periods from surgery to death, recurrence, or the last follow-up examination. The FP chemotherapy consisted of cisplatin (100 mg/m^2) on day 1 and 5-fluorouracil (750 mg/m^2/day) on days 1-5, which were administered twice (4). Surgery was performed approximately 4 to 6 weeks after the FP chemotherapy.

Assessment of pathological response to FP-NAC

The pathological responses to the FP-NAC were histopathologically determined using the surgical specimens. The response grades were based on the Japan Esophageal Society evaluation criteria (17): grade 3, no viable residual tumor cells; grade 2, two-thirds or fewer viable residual tumor cells; grade 1, two-thirds or more viable residual tumor cells; and grade 0, all residual viable tumor cells. We classified the patients with a pathological response grade of 2 or 3 as responders and those with a pathological response grade of 0 or 1 as non-responders.

Immunohistochemical staining and evaluation. The procedure used in this study has been published previously (18). Immunohistochemical staining was performed on 4-μm slices. The slides were deparaffinized with xylene and rehydrated with ethanol in graded dilutions. Antigen retrieval was performed by heating in an oven. Biopsy specimens prior to the FP-NAC were stained with anti-mouse CD24 monoclonal antibody (mAb) (clone CM323; Biocare Medical, Concord, CA, USA) at a dilution of 1:100, anti-mouse CD44 mAb (clone Ab6124; Abcam, Cambridge, UK) at a dilution of 1:100, and anti-mouse CD133 mAb (clone AC133; Miltenyi Biotec, Auburn, CA, USA) at a dilution of 1:10.

For immunostaining, 171 biopsy specimens prior to the FP-NAC were stained using the mAbs against CD44, CD133, or CD24. Tumor cells were determined positive when the circumferential membranes and cytoplasm were stained. The frequencies of tumor cells positive for the tested markers were calculated in each biopsy specimen. Subsequently, the cases were subclassified into the $CD44^{high}$ expression group (≥50% immunostained tumor cells) and the $CD44^{low}$ expression group (<50% immunostained tumor cells). In a similar manner, the cases were also subclassified into the $CD133^{high}$ expression group (≥1% immunostained tumor cells) and the $CD133^{low}$ expression group (<1% immunostained tumor cells) as well as the $CD24^{high}$ expression group (≥10% immunostained tumor cells) and the $CD24^{low}$ expression group (<10% immunostained tumor cells) (19-21).

Statistical analysis. Statistical analysis was performed using JMP software (SAS Institute Inc, Cary, NC, USA). The Kaplan–Meier method was used to plot survival curves, and the log-rank test was used to determine statistical differences. Fisher's exact test and the unpaired two-tailed *t*-test were used to test the relationships among categorical tumor variables. To investigate the risk factors associated with pathological response in the surgical specimens, logistic regression models were constructed to calculate the odds ratios (OR) and 95% confidence intervals (CI). Variables generally

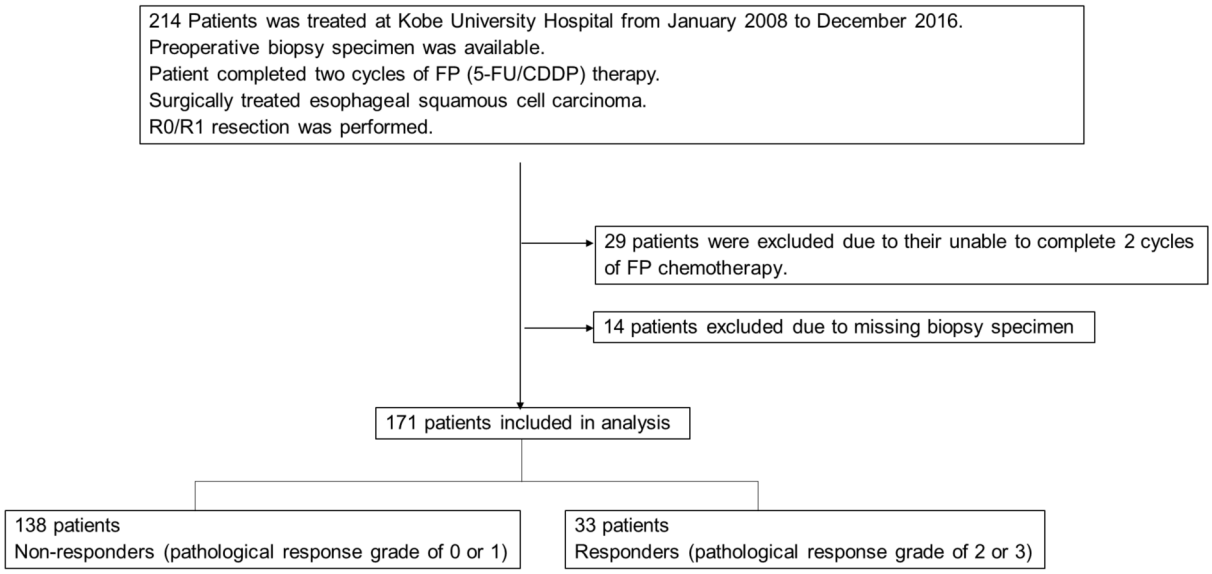

Figure 1. *Flowchart of the study cohort showing exclusions. The subjects were 214 patients with squamous cell carcinoma of the esophagus who underwent surgery after preoperative chemotherapy at our department between 2008 and 2016. From these patients, 171 were included and 43 nonqualifying cases were excluded (29 patients who failed to complete two courses of chemotherapy with a combination of 5-fluorouracil and cisplatin and 14 patients for whom no biopsy specimens were available). The study cohort included 138 non-responders and 33 responders.*

considered to be risk factors were entered into a multivariate logistic regression analysis. A Cox proportional hazard model was used to assess the effect of assessed parameters on OS. A p-value of <0.05 was considered statistically significant.

Results

Non-responders to FP-NAC had poorer prognoses than responders. A total of 214 patients with ESCC who underwent the FP-NAC followed by radical resection at Kobe University Hospital between January 2008 and December 2016 were considered for this study. We analyzed a cohort of 171 patients who underwent radical resection after completing two cycles of the FP-NAC based on our criteria. The study cohort included 138 non-responders and 33 responders (Figure 1).

The patients' characteristics and statistical analysis, stratified by the non-responders and the responders, are summarized in Table I. The patients' characteristics (age, sex, American Society of Anesthesiologists score, and clinical stage) did not differ significantly between the groups (Table I).

The Kaplan–Meier curves based on the pathological responses to the FP-NAC showed that the non-responders significantly correlated with poorer OS (p<0.0001) and relapse-free survival (RFS) (p<0.0001) compared with the responders (Figure 2).

$CD44^{high}$ and $CD44^{high}/CD24^{low}$ were associated with poor pathological responses to FP NAC. Biopsy specimens prior to the FP-NAC were immunostained for CD44, CD133, and CD24, all of which have been shown to be discriminatory markers of the ESCC-CSCs. In the surgically resected ESCC tissues, the cell membrane of tumor cells was extensively stained by the anti-CD44 mAb (Figure 3). The cytoplasm and nucleus of tumor cells were also extensively stained by the anti-CD133 mAb. In contrast, the cell membrane of the tumor cells was distinctly stained by the anti-CD24 mAb. The cases were divided into two groups (high and low) based on the frequencies of tumor cells positive for the tested markers. The relationship between the expression levels of these markers and the pathological response are summarized in Table II. The non-responders had $CD44^{high}$ (84.8%, p=0.043) and $CD44^{high}/CD24^{low}$ (73.2%, p=0.006) at higher frequencies than the responders (Table II).

$CD44^{high}/CD24^{low}$ was a predictor for FP-NAC resistance in ESCC. Logistic regression models were used to identify the factors involved in poor pathological responses in the surgical specimens. Univariate and multivariate analyses were performed to identify independent predictors for the FP-NAC resistance (Table III). In the univariate analysis, $CD44^{high}/CD24^{low}$ was associated with a higher risk of the poor pathological responses (p=0.007). In the multivariate analysis, $CD44^{high}/CD24^{low}$ was found as an independent

Table I. *Patient characteristics and statistical analysis according to pathological response.*

Variable	Non-responders (n=138)	Responders (n=33)	*p*-Value
Age			0.691
<65	49	13	
≥65	89	20	
Gender			1.000
Female	20	4	
Male	118	29	
ASA score			0.693
1	56	10	
2	63	18	
¾	19	5	
Smoking			0.615
Never	27	9	
Previous	55	12	
Now	56	12	
Neutrophil count (/μl)			0.866
<5,000	96	24	
≥5,000	41	9	
Differentiation			0.572
Well	9	1	
Moderate	45	11	
Poorly	8	4	
Others/unidentified	76	17	
Location			0.396
Ce	13	5	
Ut	18	7	
Mt	54	11	
Lt	45	10	
Ae	8	0	
Clinical T*			0.252
1	15	8	
2	26	6	
3	90	18	
4	7	1	
Clinical N*			0.127
0	26	9	
1	85	22	
2	27	2	
Clinical stage			
1	11	5	0.233
2	42	13	
3	78	15	
4	7	0	

ASA: American Society of Anesthesiologists; Ce: cervical esophagus; Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus. *Tumors were classified according to the American Joint Committee on Cancer (AJCC) TNM system.

Table II. *Statistical analysis of pathological response grade and the combination of three stem cell markers.*

Factor	Non-responders (n=138)	Responders (n=33)	*p*-Value
CD44			0.043
Low	21 (15.2%)	10 (30.3%)	
High	117 (84.8%)	23 (69.7%)	
CD133			0.266
Low	126 (91.3%)	28 (84.8%)	
High	12 (8.7%)	5 (15.2%)	
CD24			0.075
Low	115 (83.3%)	23 (69.7%)	
High	23 (16.7%)	10 (30.3%)	
$CD44^{high}/CD133^{high}$			0.604
No	129 (93.5%)	30 (90.9%)	
Yes	9 (6.5%)	3 (9.1%)	
$CD44^{high}/CD24^{low}$			0.006
No	37 (26.8%)	17 (51.5%)	
Yes	101 (73.2%)	16 (48.5%)	
$CD133^{high}/CD24^{low}$			0.087
No	132 (95.7%)	29 (87.9%)	
Yes	6 (4.3%)	4 (12.1%)	
$CD44^{high}/CD133^{high}/CD24^{low}$			0.273
No	132 (95.7%)	30 (90.9%)	
Yes	6 (4.3%)	3 (9.1%)	

risk factor for the poor pathological responses (adjusted OR=0.30, 95% CI=0.13-0.67, p=0.003).

$CD44^{high}/CD24^{low}$ was a predictor for poor survival in ESCC. The correlation of clinicopathological variables with the status of $CD44^{high}/CD24^{low}$ was examined. Histopathological venous infiltration was significantly higher in the $CD44^{high}/CD24^{low}$ group than that in the counterpart group (p<0.001). No significant differences were found in the other factors (Table IV). Survival analyses of the ESCC patients showed that, among all the combinations of the three CSC markers, the $CD44^{high}/CD24^{low}$ expression was best associated with the poor prognosis. The Kaplan–Meier curves of the patients' OS are shown in Figure 4. The median follow-up period was 94 months. There were significant differences in the OS between the $CD44^{high}/CD24^{low}$ group and the counterpart group. The 5-year OS rates were 39.6% in the $CD44^{high}/CD24^{low}$ group and 68.9% in the counterpart group (p=0.018; Figure 4A). There were no significant differences between the groups in the 5-year RFS rates (p=0.243; Figure 4B).

Univariate and multivariate analyses by Cox proportional hazard models for OS and RFS were performed to identify independent predictors for the patients' survival based on the immunostaining data of the biopsy specimens prior to the FP-NAC (Tables V and VI). In the multivariate analysis, cT stage (p<0.0001) and $CD44^{high}/CD24^{low}$ (p=0.013) were independent factors associated with the poor OS (Table V). Also, cT stage (p<0.0001) was an independent factor associated with poor RFS (Table VI).

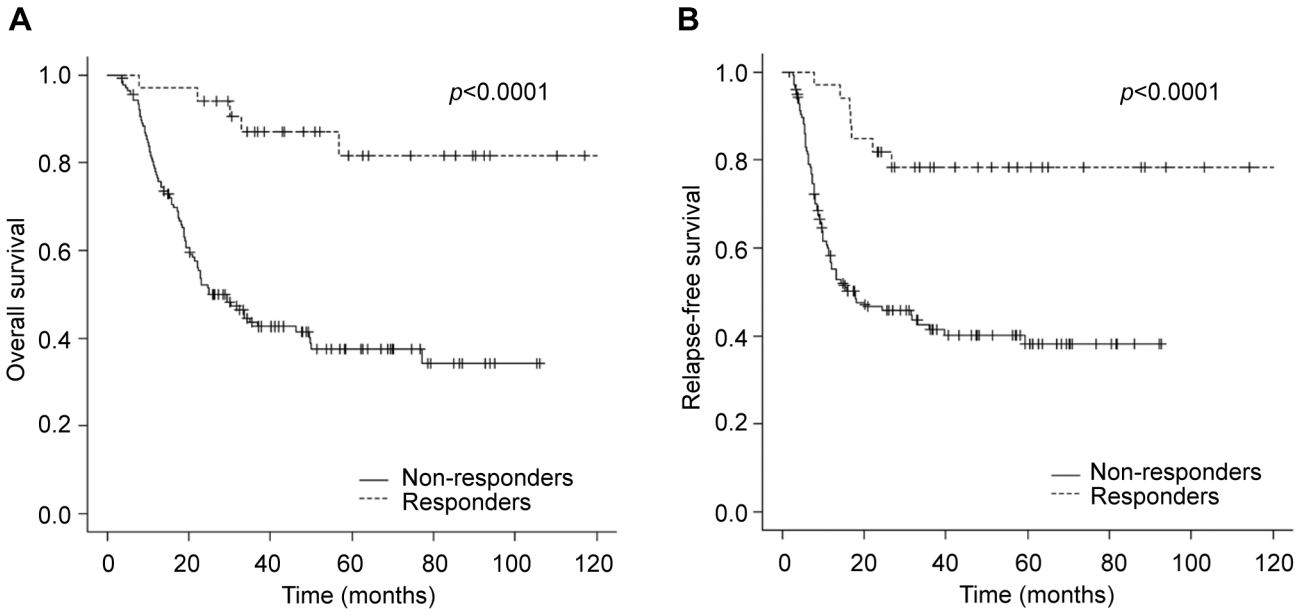

Figure 2. *Survival analysis of pathological response after neoadjuvant chemotherapy with a combination of 5-fluorouracil and cisplatin in esophageal squamous cell carcinoma. Non-responders were significantly correlated with worse prognoses. The p-values were determined by log-rank tests.*

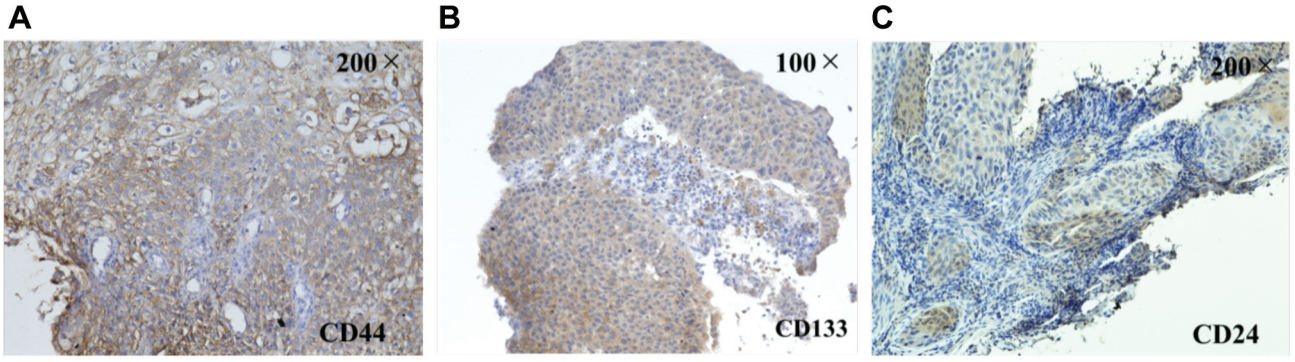

Figure 3. *Immunohistochemical staining of esophageal squamous cell carcinoma tissue for CD44, CD133, and CD24. (A) Membrane staining using CD44 antibody. (B) Membrane staining using CD133 antibody. (C) Membrane staining using CD24 antibody.*

Discussion

In this study, we clarified that $CD44^{high}/CD24^{low}$ expression in the biopsy specimens of ESCC prior to the FP-NAC was a predictor of the therapy resistance and poor prognosis of the ESCC patients. We analyzed a cohort of 171 patients who underwent radical resection after completing two cycles of the FP-NAC based on our criteria (Figure 1). Consistent with the previous studies (22), the non-responders to the FP-NAC had poorer prognoses than the responders in OS and RFS (Figure 2). The biopsy specimens prior to the FP-NAC were immunohistochemically analyzed for the ESCC-CSC markers (Figure 3). Regarding the relationship between the expression levels of these markers and the pathological responses of the surgical specimens to the FP-NAC, the frequencies of $CD44^{high}$ and $CD44^{high}/CD24^{low}$ were significantly greater in the non-responders than the responders (Table II). The multivariate analysis of the pathological responses showed that $CD44^{high}/CD24^{low}$ was an independent predictor of the poor responses to the FP-NAC (Table III). In terms of OS and RFS, $CD44^{high}/CD24^{low}$ conferred a poorer prognosis than the

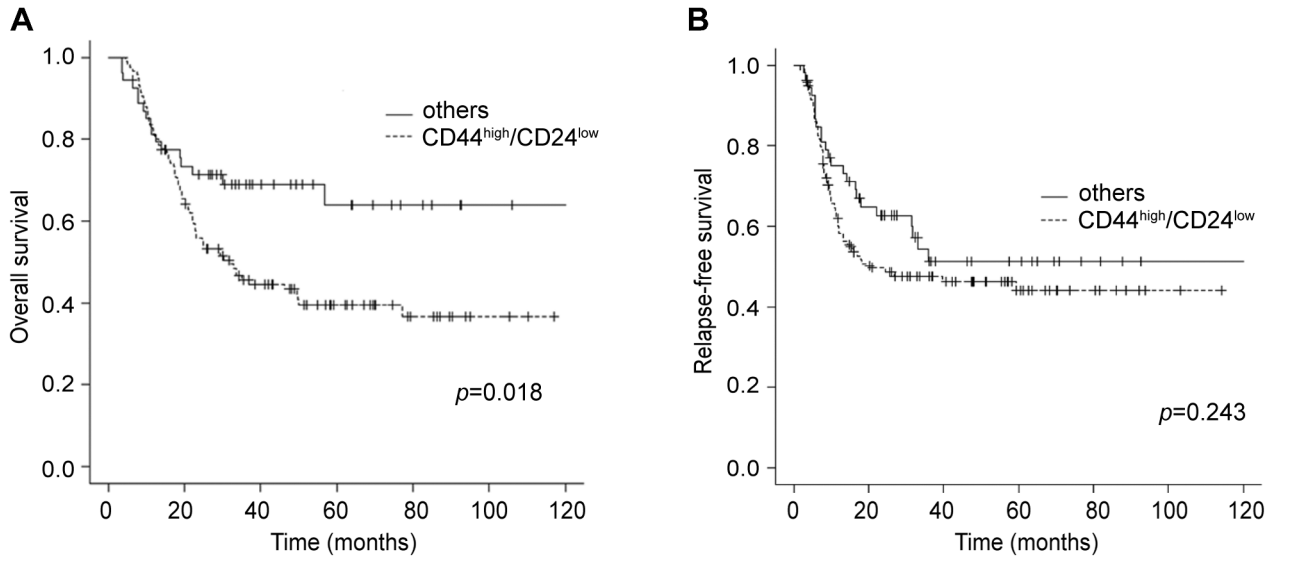

Figure 4. *Survival analyses based on CD44^{high}/CD24^{low} expression. CD44^{high}/CD24^{low} in pretreatment biopsy specimens is a feasible prognostic factor for radical resection after neoadjuvant chemotherapy with a combination of 5-fluorouracil and cisplatin in esophageal squamous cell carcinoma. The p values were determined by log-rank tests. (A) Patients with CD44^{high}/CD24^{low} expression had significantly shorter overall survival (p=0.018). (B) CD44^{high}/CD24^{low} expression was not associated with relapse-free survival (p=0.243).*

Table III. *Logistic regression analysis of pathological response grade.*

Factor	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	*p*-Value	Odds ratio (95% CI)	*p*-Value
Age ≥65/<65	0.85 (0.39-1.85)	0.677		
Gender (male/female)	1.23 (0.26-2.56)	0.725		
ASA score ≥3/<3	1.12 (0.38-3.25)	0.837		
BMI ≤19/>19	0.58 (0.24-1.39)	0.221		
Smoking +/–	0.65 (0.27-1.55)	0.332		
Hypertension +/–	1.30 (0.61-2.79)	0.496		
Diabetes +/–	2.33 (0.80-6.77)	0.119	3.00 (0.97-9.33)	0.057
Respiratory dysfunction +/–	1.23 (0.59-2.70)	0.552		
WBC >8,000/<8,000	0.8 (0.30-2.12)	0.653		
Hb >10.0/<10.0	0.68 (0.29-1.64)	0.393		
CEA ≥5.0/<5.0	0.46 (0.15-1.40)	0.170	0.31 (0.09-1.02)	0.055
SCC ≥1.5/<1.5	0.96 (0.44-2.06)	0.913		
cT* ≥3	0.57 (0.26-1.25)	0.163	0.48 (0.21-1.10)	0.083
cN* ≥1	0.62 (0.26-1.49)	0.284		
cM* ≥1	1.02 (0-inf)	0.991		
CD44^{high}/CD24^{low} +/–	0.34 (0.16-0.75)	0.007	0.30 (0.13-0.67)	0.003

CI: Confidence interval; ASA: American Society of Anesthesiologists; BMI: body mass index; WBC: white blood cell; Hb: hemoglobin; CEA: carcinoembryonic antigen; SCC: squamous cell carcinoma antigen. *Tumors were classified according to the American Joint Committee on Cancer (AJCC) TNM system.

counterpart groups, as expected (Figure 4). Moreover, CD44^{high}/CD24^{low} was found to be an independent predictor of poor prognosis for OS (Tables V and VI).

The clinical value of the predictors for the NAT responders depends on when the prediction can be made: before NATs, after NATs, or after surgery. Previous studies have reported that the pathological responses to the NATs is one of the most reliable predictors of the prognosis after surgery (22, 23). Therefore, we aimed at determining the biopsy specimen-derived predictors of pathological

Table IV. *Correlation between clinicopathological variables and CD44^{high}/CD24^{low}.*

Factor	Markers		*p*-Value
	Others	CD44^{high}/CD24^{low}	
Age			0.732
<65	21	41	
≥65	33	76	
Gender			1.000
Female	7	17	
Male	47	100	
ASA score			0.681
1	20	46	
2	25	56	
3	9	13	
4	0	2	
Smoking			0.829
Never	11	25	
Previous	23	44	
Current	20	48	
Histology			0.407
Well	11	21	
Moderate	19	56	
Poor	8	16	
Unidentified	16	24	
pT*			0.087
0	6	5	
1	19	27	
2	6	14	
3	18	62	
4	5	9	
pN*			0.867
0	22	45	
1	18	34	
2	8	22	
3	6	16	
ly			0.234
0	34	62	
≥1	18	53	
Unidentified	2	2	
v			0.001
0	38	51	
≥1	14	64	
Unidentified	2	2	

ASA: American Society of Anesthesiologists; ly: lymphovascular invasion; v: venous invasion. *Tumors were classified according to the American Joint Committee on Cancer (AJCC) TNM system.

responses to the FP-NAC. Predicting the therapeutic effects prior to the NATs would considerably benefit patients to personalize treatment options and avoid toxicity. If the FP-NAC is not chosen for ESCC, other NATs such as FP-based chemoradiotherapy, FP plus docetaxel, FP plus docetaxel-based chemoradiotherapy, or surgery alone are envisioned; the problem is that adequate evidence-based alternatives are lacking (24, 25). If the treatment options for the FP chemotherapy-resistant patients are improved (such as immune checkpoint inhibitors and molecular-targeted therapies) (26), our results will contribute to the personalized selection of NATs for ESCC by precision medicine.

Biopsy specimens of ESCC prior to the NATs are always helpful as a source to identify therapeutic predictors with no additional intervention. In fact, in this study, we evaluated the combinations of CSC markers in the biopsy specimens prior to treatment with FP-NAC and successfully demonstrated that the immunostaining status of CD44^{high}/CD24^{low} was a valuable predictor of the FP-NAC resistance and the poor prognosis of ESCC patients. Similarly, some studies have shown that the expression levels of the recombinase RAD51 and ASK1-interacting protein-1 (AIP1) in biopsy specimens prior to treatment with NATs would be useful as predictors of the NAT responders (27, 28). Of note is that immunostaining of biopsy specimens does not always go well. For example, the expression levels of PD-L1, which is related to the therapeutic effects of immune checkpoint inhibitors (29, 30), may not be accurately quantified in the biopsy specimens due to its tumoral heterogeneity. We need to take the characteristics of specimens and markers of interest into careful consideration particularly when we handle biopsy specimens.

CSCs are considered as the most significant targets to overcome therapy resistance of tumors. Therefore, we hypothesized that the ESCC-CSC markers would be good predictors of the FP-NAC resistance. Several reports have indicated that CD44 alone is useful as an ESCC-CSC marker (10, 31). However, ESCCs have been shown to express very high levels of CD44 (10, 11), which suggest that CD44 alone is insufficient to specify ESCC-CSCs. In this study, the CD44^{high} group, in which ≥50% of the tumor cells were positively immunostained for CD44, was correlated with therapy resistance (Table II) and accounted for 81.9% of all the cases. Consistent with the previous studies (10, 11), we concluded that CD44 alone would be insufficient as a clinical predictor. Therefore, we investigated combinations of CD44 with other markers to specify ESCC-CSCs more precisely. As a result, we found CD44^{high}/CD24^{low} as a negative predictor of the therapeutic responses to FP-NAC. Here, CD24 is a heat-stable antigen expressed in some types of cancers and a marker for the diagnosis of tumorigenesis and is associated with cell adhesion and migration (32). From a viewpoint of ESCC-CSCs, however, CD24^{-} appears to be involved in the CSC biology for the following reasons. In a previous study, the CD44^{+}CD24^{-} subpopulations in pre-chemotherapy biopsy specimens of esophageal cancer have been shown to have stemness and radiotherapy resistance and be a predictor of chemoradiotherapy responses (33). In this regard, although CD44^{high}/CD24^{low} does not directly indicate the presence of the CD44^{+}CD24^{-} CSCs, it is highly likely that this population contains the CSCs abundantly (33). Taken together, the data in this study successfully

Table V. *Analysis of overall survival*.

Factor	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
Age ≥65/<65	1.31 (0.83-2.07)	0.299		
Gender (male/female)	1.11 (0.60-2.04)	0.713		
ASA score ≥3/<3	1.15 (0.62-2.11)	0.693		
BMI ≤19/>19	1.73 (1.12-2.68)	0.009	1.15 (0.70-1.89)	0.568
Smoking +/–	1.42 (0.81-2.48)	0.203		
Hypertension +/–	0.74 (0.48-1.14)	0.140		
Diabetes +/–	0.56 (0.24-1.29)	0.162		
Respiratory dysfunction +/–	1.21 (0.79-1.86)	0.321		
WBC >8,000/<8,000	1.09 (0.65-1.81)	0.750		
Hb >10.0/<10.0	1.48 (0.95-2.31)	0.086		
CEA ≥5.0/<5.0	1.60 (0.98-2.63)	0.062		
SCC ≥1.5/<1.5	1.90 (1.24-2.92)	0.003	1.60 (0.97-2.63)	0.658
cT* ≥3	3.58 (2.02-6.37)	<0.0001	5.42 (2.44-12.03)	<0.0001
cN* ≥1	1.58 (0.88-2.87)	0.125		
cM* ≥1	3.84 (1.65-8.93)	0.002	2.11 (0.81-5.51)	0.127
$CD44^{high}/CD24^{low}$ +/–	2.00 (1.16-3.45)	0.020	2.21 (1.18-4.14)	0.013

HR: Hazard ratio; CI: confidence interval; ASA: American Society of Anesthesiologists; BMI: body mass index; WBC: white blood cell; Hb: hemoglobin; CEA: carcinoembryonic antigen; SCC: squamous cell carcinoma antigen. *Tumors were classified according to the American Joint Committee on Cancer (AJCC) TNM system.

Table VI. *Analysis of relapse-free survival*.

	Univariate analysis		Multivariate analysis	
Factor	HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
Age ≥65/<65	1.22 (0.78-1.93)	0.384		
Gender (male/female	1.06 (0.57-1.95)	0.852		
ASA ≥3/<3 score	1.00 (0.51-1.93)	0.992		
BMI ≤19/>19	1.62 (1.04-2.53)	0.034	1.19 (0.76-1.89)	0.448
Smoking +/–	1.06 (0.64-1.77)	0.822		
Hypertension +/–	0.76 (0.49-1.17)	0.208		
Diabetes +/–	0.46 (0.19-1.14)	0.092		
Respiratory dysfunction +/–	1.19 (0.77-1.83)	0.437		
WBC >8,000/<8,000	1.13 (0.67-1.90)	0.649		
Hb >10.0/<10.0	1.22 (0.77-1.94)	0.400		
CEA ≥5.0/<5.0	1.08 (0.62-1.86)	0.796		
SCC ≥1.5/<1.5	1.55 (1.00-2.38)	0.048	1.29 (0.82-2.02)	0.274
cT* ≥3	2.72 (1.68-4.40)	<0.0001	3.24 (1.80-5.85)	<0.0001
cN* ≥1	1.59 (0.88-2.88)	0.123		
cM* ≥1	3.30 (1.33-8.25)	0.010	3.24 (1.80-5.85)	0.172
$CD44^{high}/CD24^{low}$ +/–	1.33 (0.82-2.15)	0.246		

HR: Hazard ratio; CI: confidence interval; ASA: American Society of Anesthesiologists; BMI: body mass index; WBC: white blood cell; Hb: hemoglobin; CEA: carcinoembryonic antigen; SCC: squamous cell carcinoma antigen. *Tumors were classified according to the American Joint Committee on Cancer (AJCC) TNM system.

proved our original hypothesis that the presence of CSCs in tumor tissues may be the cause of treatment resistance and poor prognosis. Further studies are needed to identify accurate markers of ESCC-CSC.

In conclusion, this study shows that, as ESCC-CSC combination markers, the expression levels of $CD44^{high}/CD24^{low}$ in the biopsy specimens prior to the FP-NAC is a valuable predictor of FP-NAC resistance and poor prognosis.

Our data may clinically enable us to identify ESCC patients who do not benefit from the FP-NAC and should choose other treatment strategies. Also, our data may contribute to elucidating the mechanism of chemoresistance of ESCC-CSCs.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

KA, AN, and KY conceived and planned the study. AN, KA, KY conducted the experiments. KA, AN, KY, and MF, contributed to the interpretation of the results. KA took the lead in writing the manuscript. KY, MF. MS, JM and YK critically revised the manuscript for intellectual content. All Authors provided critical feedback and helped shape the research, analysis, and manuscript.

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