CD24 and CK4 are upregulated by SIM2, and are predictive biomarkers for chemoradiotherapy and surgery in esophageal cancer

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Abstract. Definitive chemoradiotherapy (CRT) is a less invasive therapy compared with surgery for some types of cancer; however, the 5-year survival rate of patients with stages II-III esophageal squamous cell carcinoma (ESCC) is only 37%. Therefore, prediction of CRT responders is necessary. Unfortunately, no definitive biomarker exists that is useful to predict survival outcome following CRT. From our previous microarray study, *CD24* and keratin 4 (*KRT4*), which encodes cytokeratin 4 (CK4), were overexpressed in the favorable prognostic epithelial subtype with SIM bHLH transcription factor 2 (SIM2) expression. This study investigated the association between their mRNA and protein expression levels, and clinicopathological characteristics, and also investigated the functions of *CD24* in SIM2-mediated tumor differentiation and CRT sensitivity. High *CD24* and *KRT4* mRNA expression was associated with a favorable prognosis following CRT. Multivariate analyses revealed that high CD24 and CK4 protein expression, as determined by immunohistochemistry, and differentiated type were independent factors for predicting a favorable prognosis in response to CRT. Notably, in cases with low CD24 or CK4, surgery was suggested to be a good therapeutic modality compared with CRT. *CD24* and *KRT4* were expressed preferentially in differentiated layers of the normal esophageal mucosa, and their mRNA expression in 3D cultured ESCC cells was induced by *SIM2* transfection, thus suggesting that *CD24* and *KRT4* were downstream differentiation markers of SIM2. Furthermore, *CD24* small interfering RNA increased the mRNA expression levels of superoxide dismutase 2 and enhanced H_2O_2 resistance, thus indicating the involvement of CD24 in the radiosensitivity of patients with ESCC; however, it had no effect on cisplatin sensitivity. In conclusion, the two markers CD24 and CK4 may be considered predictive biomarkers for definitive CRT.

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Introduction

Esophageal cancer is the sixth most common cause of cancer-associated mortality worldwide (1). Neoadjuvant chemoradiotherapy (CRT) followed by esophagectomy is the standard treatment for locally advanced esophageal squamous cell carcinoma (ESCC) in Western countries, whereas neoadjuvant chemotherapy (CT) followed by esophagectomy or definitive CRT (CRT alone as a primary therapy) are the standard treatments in Japan (2). Although neoadjuvant CT and definitive CRT improve the prognosis of patients with ESCC, the 5-year survival rate is still 37-55% (2,3). Local recurrence and metastasis are major causes of poor prognosis. Nevertheless, the prediction is difficult, creating a need for predictive factors that select patients who are potentially curable with definitive CRT.

By comparing microarray profiles among pre- and post-treatment biopsy specimens of patients with ESCC, our previous study identified a good responder subtype with cytotoxic T-lymphocyte signatures that were activated by

CRT (4). Clustering analysis of 234 tumor immunity-related genes in 121 pre-treatment ESCC specimens distinguished the immune-activated cases, termed I-type, from other cases. In the I-type, the clinical outcome of cadherin 2 (CDH2)-negative cases was significantly better than that of the CDH2-positive cases. Notably, *CD24*, keratin 4 (*KRT4*) and SIM bHLH transcription factor 2 (*SIM2*) were overexpressed in the CDH2-negative cases (4). The differentiation degree in squamous cell carcinoma has been reported to influence sensitivity and prognosis in response to CRT (5,6). SIM2 is a member of the basic HLH-PER-ARNT-SIM transcription factors, which is isolated from a Down's syndrome-crucial region (7-9). Aberrant SIM2 expression has been reported in several types of cancer (10,11). Recently, we identified SIM2 as a predictive biomarker for patients with cervical cancer who were potentially curable with CRT (12). Furthermore, our previous study reported that SIM2 in ESCC might be a key transcription factor involved in tumor differentiation and CRT sensitivity through downregulation of DNA repair and antioxidant genes. Therefore, SIM2 may be associated with the response to definitive CRT (13).

CD24 is a small mucin-like cell surface protein, which is expressed on lymphocytes and epithelial cells (14), and is also expressed in various types of cancer, including colorectal, pancreatic, lung, liver, ovarian and breast cancer (15-18). These studies also reported that CD24 overexpression is associated with an aggressive course of the disease. Furthermore, CD24 may serve a role in the metastasis of breast cancer (19-21), cervical cancer (18), gastric cancer (22) and bladder cancer (23,24). CD24 has also been reported as a marker for stem cells in pancreatic and ovarian cancer (25,26). However, the role of CD24 in ESCC remains obscure.

KRT4 encodes a type II cytokeratin, cytokeratin 4 (CK4), which is specifically found in differentiated layers of the esophageal epithelia. *KRT4* is downregulated in ESCC and head and neck squamous cell carcinoma compared with in normal squamous epithelium (27,28). Its low expression is associated with local recurrence of head and neck squamous cell carcinoma (29). However, the biological functions and clinical significance of CK4 and CD24 remain unknown in ESCC. This study investigated the association between their mRNA and protein expression levels, and clinicopathological characteristics, and also investigated the functions of *CD24* in SIM2-mediated tumor differentiation and CRT sensitivity.

Materials and methods

Clinical samples. Patients with ESCC who received definitive CRT or curative esophagectomy with extended lymph node dissection (surgery) as an initial treatment at the National Cancer Center Hospital East (Kashiwa, Japan) between June 2005 and March 2009 were recruited. The eligibility criteria were as follows: i) Patients pathologically diagnosed, using biopsy specimens, with squamous cell carcinoma prior to receiving definitive CRT or surgery; ii) patients with stage II/III ESCC who underwent definitive CRT or surgery; and iii) patients <75 years old whose performance status according to the Eastern Cooperative Oncology Group was 0.1 (30). Clinical staging before neoadjuvant CT (in the surgery group) or definitive CRT was determined according to the Union for International Cancer Control-Tumor-Node-Metastasis classification (6th edition) (31), based on endoscopic findings and contrast enhanced computed tomography (CECT). Patients with prior or concurrent types of cancer were excluded from this study. In the surgery group, clinical outcomes were determined following surgery alone or neoadjuvant CT followed by surgery. However, patients who were not able to receive a scheduled complete course of definitive CRT were included, because such patients whose therapeutic responses are unpredictable could not be excluded prior to treatment.

Cell culture. The ESCC T.Tn cell line was purchased from the Japanese Collection of Research Bioresources Cell Bank. T.Tn cells were propagated in DMEM/Ham's F-12 (Wako Pure Chemical Industries, Ltd.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and maintained at 37˚C in 95% humidified air containing 5% CO_2. A 35-mm NanoCulture Plate (SCIVAX Corporation) was used for three-dimensional (3D) culture (13).

Laser-captured micro-dissection (LCM). The human esophagus samples were embedded in TissueTek O.C.T. Compound (Sakura Finetek Japan) and snap-frozen. The cryostat sections (8 μm) were dissected using a PixCell II LCM system (Arcturus Engineering, Inc.). To avoid contamination with dysplastic or cancerous tissues, normal esophageal mucosa was obtained from gastric cancer samples with normal esophageal tissue for semi-quantitative reverse transcription-PCR (RT-PCR) analysis of the three cell layers (differentiated, parabasal and basal cell layers).

Microarray analysis. RNA was isolated from the biopsy samples from patients prior to treatment using ISOGEN lysis buffer (Nippon Gene Co., Ltd.), and were biotin-labeled followed by hybridization to microarrays (Human Genome U133 Plus 2.0 Array; Affymetrix, Inc.), according to manufacturer's protocol. The scanned data of the arrays were processed by Affymetrix Microarray Suite version 5.0 (Affymetrix, Inc.). All of the microarray data were deposited in a minimum information about a microarray experiment-compliant database, Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/); the accession number is GSE69925 (4).

RT-PCR. Total RNA was isolated from cells using ISOGEN lysis buffer (Nippon Gene Co., Ltd.) followed by precipitation with isopropanol. RT was performed using oligo dT primers from the SuperScript III First-Stand Synthesis system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. PCR was carried out using the AccuPrimeTaq DNA Polymerase system (Thermo Fisher Scientific, Inc.), within the linear range of amplification, for long isoforms of *SIM2* (24 cycles), *CD24* (23 cycles) *KRT4* (18 cycles) and β-actin (*ACTB*; 22 cycles). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by the aforementioned number of cycles at 95˚C for 1 min, 56˚C for 1 min and 72˚C for 1 min, with a final extension step at 72˚C for 10 min. PCR products were then separated by electrophoresis with 2% agarose gels and results were visualized using ethidium bromide (Wako Pure Chemical Industries, Ltd.).

RT-quantitative PCR (RT-qPCR) was carried out for long isoforms of *SIM2*, *CDH2*, vimentin (*VIM*), snail family transcriptional repressor 2 (*SNAI2*), twist family bHLH transcription factor (*TWIST*)*1, TWIST2, CD24, KRT4* and *ACTB*. In accordance with the manufacturer's protocol, RT was conducted using the SuperScript III First-Stand Synthesis system (Thermo Fisher Scientific, Rockford, IL) and qPCR was performed on a Bio-Rad iCycler with iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 55˚C for 30 sec, and a final step at 95˚C for 1 min and 55˚C for 1 min. Results are presented as linearized quantification cycle (Cq) values normalized to *ACTB* and the indicated reference value ($2^{-\Delta\Delta Cq}$) (32). Primer sequences are listed in Table I.

Plasmid transfection. The pCMV6-AC-GFP plasmid containing *SIM2* cDNA was purchased from OriGene Technologies, Inc. T.Tn cells were plated at $2x10^6$ per 10-cm dish, and transfected with either pCMV6-AC-GFP-*SIM2* or empty pCMV6-neo (OriGene Technologies, Inc.). Briefly, cells were transfected with 4 μg plasmid DNA in 10 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, overnight at 37˚C. Subsequently, the cells were plated at $6x10^5$ cells/3.5 cm NanoCulture Plate (SCIVAX Corporation).

Immunohistochemistry (IHC) and hematoxylin and eosin (HE) staining. Specimens fixed in 10% formalin at room temperature for 8-24 h and embedded in paraffin were cut into 4-μm sections, which were dewaxed and dehydrated for routine HE staining.

For IHC, the endogenous peroxidase activity of 4-μm sections were cut from paraffin-embedded specimens, and the endogenous peroxidase activity of the sections was blocked with 3% H_2O_2 in ethanol for 5 min at room temperature, followed by additional blocking with 3% BSA-PBS (Roche Diagnostics GmbH) for 1 h at room temperature. Antigen retrieval was performed in a microwave oven at 95˚C using 10 mM citrate buffer (pH 6.0) for 20 min (CD24 antigen) or Target Retrieval Solution (cat. no. S2367; Dako; Agilent Technologies, Inc.; pH 9.0) for 10 min (CK4 antigen). Anti-CD24 (1:500; cat. no. NB100-64861; Novus Biologicals, LLC) and anti-CK4 antibodies (1:500; cat. no. ab9004; Abcam) were diluted at 1:500 and slides were incubated with them at 4˚C overnight. The slides were then incubated with a horseradish peroxidase (HRP)-labeled secondary antibody (Envision™ Kit/HRP system; cat. No. K4063; Dako; Agilent Technologies, Inc.) at room temperature for 30 min and visualized by DAB (DAB+ Liquid; Dako; Agilent Technologies, Inc.). The positive percentage of cancer cells for each case was determined by a pathologist who was blinded to the clinical data. IHC and HE staining were detected under a Nikon ECLIPSE light microscope (Nikon Corporation) and was analyzed using NIS-Elements BR version 4.10 software (Nikon Corporation).

Small interfering RNA (siRNA) transfection. *CD24* siRNAs and control siRNA (cat. no. AM4635) were purchased from Ambion; Thermo Fisher Scientific, Inc. The sequences were as follows: siRNA s2615, UCAAGUAACUCCUCCCAGAtt; siRNA s2616, CCAGAGUACUUCCAACUCUtt). siRNAs (75 nM) were introduced into $4x10^5$ T.Tn cells (50% cell confluence) using DharmaFECT 1 Transfection Reagents (GE Healthcare Dharmacon, Inc.) and cells were incubated for 3 days at 37˚C.

Western blotting. Cells were lysed in Laemmli Sample buffer (Bio-Rad Laboratories, Inc.) containing DTT and 1% protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA), and protein concentration was analyzed using the Protein Quantification Assay (MACHEREY-NAGEL GMBH & Co. KG). Protein samples (35 μg) were separated by electrophoresis using a NovexWedge Well 4-20% Tris-Glycine Gel (Thermo Fisher Scientific, Inc.). Proteins were transferred to nitrocellulose membranes, which were blocked with 5% Membrane Blocking Reagent (cat. no. RPN2125; GE Healthcare) for 1 h at room temperature, and incubated with anti-CD24 (1:200; cat. no. sc-58999; Santa Cruz Biotechnology, Inc.) at 4˚C overnight or with anti-β-actin (1:3,000; cat. no. 4967; Cell Signaling Technology, Inc.) at room temperature for 2 h. The membranes were then washed and incubated with HRP-conjugated anti-mouse immunoglobulin (1:3,000; cat. no. P0260; Dako; Agilent Technologies, Inc.) or HRP-conjugated anti-rabbit immunoglobulin (1:3,000; cat. no. P0399; Dako; Agilent Technologies, Inc.) at room temperature for 2 h. Bands were visualized with Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.).

H_2O_2 or cisplatin (CDDP) treatment. Cells were plated at $1x10^4$ cells/well in a 96-well NanoCulture Plate (SCIVAX Corporation) after siRNA transfection. A total of 1 day after plating, cells were treated with H_2O_2 (150 μM; Wako Pure Chemical Industries, Ltd.) or CDDP (5 μM; Sigma-Aldrich; Merck KGaA) at 37˚C for 1 or 3 days, respectively. The number of viable cells was counted using a CellTiter-Glo Luminescent Cell Viability Assay (Promega Corporation), according to the manufacturer's protocol.

TGF-β treatment. T.Tn cells were plated at $8x10^5$ cells/well in a 6-well plate and were incubated at 37˚C overnight. Subsequently, the cells were treated with TGF-β1 (10 ng/ml; R&D Systems, Inc.) at 37˚C for 3 days.

Statistical analysis. RT-qPCR data are expressed as the mean ± SE and were analyzed using one-way ANOVA followed by Tukey's honestly significant difference test or Dunnett's multiple comparison test. Recurrence-free survival (RFS) and overall survival (OS) were estimated using the Kaplan-Meier method and were compared using the log-rank test by GraphPad Prism version 7.0a (GraphPad Software, Inc.). RFS was defined as the period from the date of definitive CRT or surgery until the date of death or recurrence, which was clinically confirmed through endoscopy or CECT. OS was defined as the time from the date of definitive CRT or surgery until the last confirmed date of survival or death, regardless of the cause of death. Multivariate analysis with the Cox model was used to investigate the association between patient background, endoscopic findings and clinicopathological factors, including death or recurrence. IBM SPSS statistical software package (version 22.0 for Mac; IBM Japan Ltd.) and Ekuseru-Toukei

Table I. Primer sequences for reverse transcription-PCR.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
ACTB	GAAGTCCCTTGCCATCCTAA	GCACGAAGGCTCATCATTCA
CD24	GCCTCGACACACATAAACCT	CTGTTCGATCTGTTTGTTCC
SIM2[a]	TGCCAACCCTGTGTCACTTA	ACCCTCGGCTTATTTCCTGT
SIM2[b]	CTTCCCTCTGGACTCTCACG	AGGCTGTGCCTAGCAGTGTT
KRT4	CAGGAGTGTCATCTCCAGAA	GAAGATTCACCTGCAGATGG
SNAI2	TAGGAAGAGATCTGCCAGAC	CCCCAAGGCACATACTGTTA
VIM	GCTTTCAAGTGCCTTTCTGC	GTTGGTTGGATACTTGCTGG
CDH2	GGCATAGTCTATGGAGAAGT	GATTTCACAAGTCTTCACCTG
TWIST1	GCATTTTACCATGGGTCCTC	ATACTGGGATCAAACTGGCC
TWIST2	GAGCCTCTGCATGATTGTTTC	CACTGCAGTCACTTAGCTTG
SOD2	ATGATCCCAGCAAGATAATG	AGGACCTTATAGGGTTTTCAG

[a]Long mRNA isoform, this primer was used to detect the mRNA expression in three layers of normal esophageal mucosa; [b]short and long mRNA isoforms, this primer was used to detect the mRNA expression in plasmid- and siRNA-transfected T.Tn cells. *ACTB*, β-actin; *CDH2*, cadherin 2; *KRT4*, keratin 4; *SNAI2*, snail family transcriptional repressor 2; *VIM*, vimentin.

2010 (Social Survey Research Information Co., Ltd.) were used for statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

CD24 and KRT4 are differentiation markers that are downstream of SIM2. Initially, this study analyzed the semi-quantitative RT-PCR of *CD24*, *KRT4* and *SIM2* in three layers (differentiated, parabasal and basal cell layers) of normal esophageal mucosa (23 cycles for *CD24*, 18 cycles for *KRT4* and 24 cycles for *SIM2*). *CD24* and *KRT4* were highly expressed in differentiated cell layers and moderately expressed in parabasal cell layers. *SIM2* was highly expressed in parabasal and basal cell layers, and moderately expressed in differentiated cell layers (Figs. 1A and S1). Subsequently, CD24, CK4 and SIM2 protein expression was detected in normal esophageal mucosa by IHC. In accordance with the RT-PCR results, CD24 and CK4 were highly expressed in differentiated and parabasal cell layers, whereas SIM2 was expressed highly in parabasal and basal cell layers (Fig. 1B). These data suggested that CD24 and CK4 are differentiation markers in the stratified squamous epithelia of the esophagus.

To investigate whether *CD24* and *KRT4* are downstream genes of the tumor differentiation-inducer *SIM2*, a 3D culture system was used, which has been reported to induce differentiation of ESCC through adhesion restriction (13). Overexpression of *SIM2* in T.Tn cells followed by 3D culture has been reported to increase spheroid formation (13); in this study, *SIM2* overexpression and 3D culture significantly increased *CD24* and *KRT4* mRNA expression at day 8 (Fig. 1C). These results of *in vitro* 3D cell culture suggested that *CD24* and *KRT4* may be downstream differentiation markers of *SIM2*.

Patients with ESCC and high CD24 and KRT4 mRNA expression exhibit a favorable prognosis with definitive CRT. Clinicopathological characteristics of patients with ESCC who received definitive CRT (n=81) or surgical resection (n=63) are shown in Table SI. Using our previously obtained microarray data (GSE69925) (4), *CD24* and *KRT4* mRNA expression was examined in biopsy specimens from 81 patients with ESCC (clinical stages II and III) prior to definitive CRT. A total of 15 of the 81 cases (18.5%) were classified into a high *CD24* mRNA expression group, whose *CD24* expression was higher than mean + SD (Fig. 2A). Similarly, 22 of the 81 cases (27%) were classified into a high *KRT4* mRNA expression group, whose *KRT4* expression signal intensity was >50,000 (Fig. 2A). Kaplan-Meier analysis revealed that RFS and OS of the high *CD24* or *KRT4* mRNA expression groups were significantly longer than those of the low *CD24* or *KRT4* mRNA expression groups (*CD24*, lower than mean-SD and *KRT4*, signal intensity was <1,000) (Fig. 2B and C).

Immunohistochemical analyses for predicting patients with ESCC with a favorable prognosis following definitive CRT. According to the microarray data, *CD24* and *KRT4* mRNA expression may be candidate markers for predicting patients with ESCC with a favorable prognosis in response to definitive CRT. The *CD24* and *KRT4* genes encode CD24 and CK4 proteins, respectively. To verify the results of microarray analysis, each of these two marker proteins was examined by immunohistochemical staining in biopsy specimens obtained from 81 patients with ESCC prior to definitive CRT. Representative data are shown in Fig. 3A. According to the cut-off values for CD24 and CK4 positivity rates, a sensitivity test was performed using the hazard ratio (HR) for OS. The minimum HR was obtained when the cut-off values of 20% CD24-positive and 10% CK4-positive in tumor cells were adopted (CD24: HR, 0.446; 95% CI, 0.219-0.909; P=0.026 and CK4: HR, 0.176; 95% CI, 0.042-0.728; P=0.016). High CD24 expression was detected in 26 of the 81 patients (32%), whereas high CK4 expression was detected in 14 of the 81 patients (17%) (Table II). As shown in Fig. 3B, RFS and OS of patients with ESCC and high CD24 or CK4 protein expression were significantly higher than those of patients with ESCC and low CD24 or CK4 protein expression. Only 10 patients with ESCC exhibited high expression of both CD24 and CK4, whereas 71 patients with ESCC

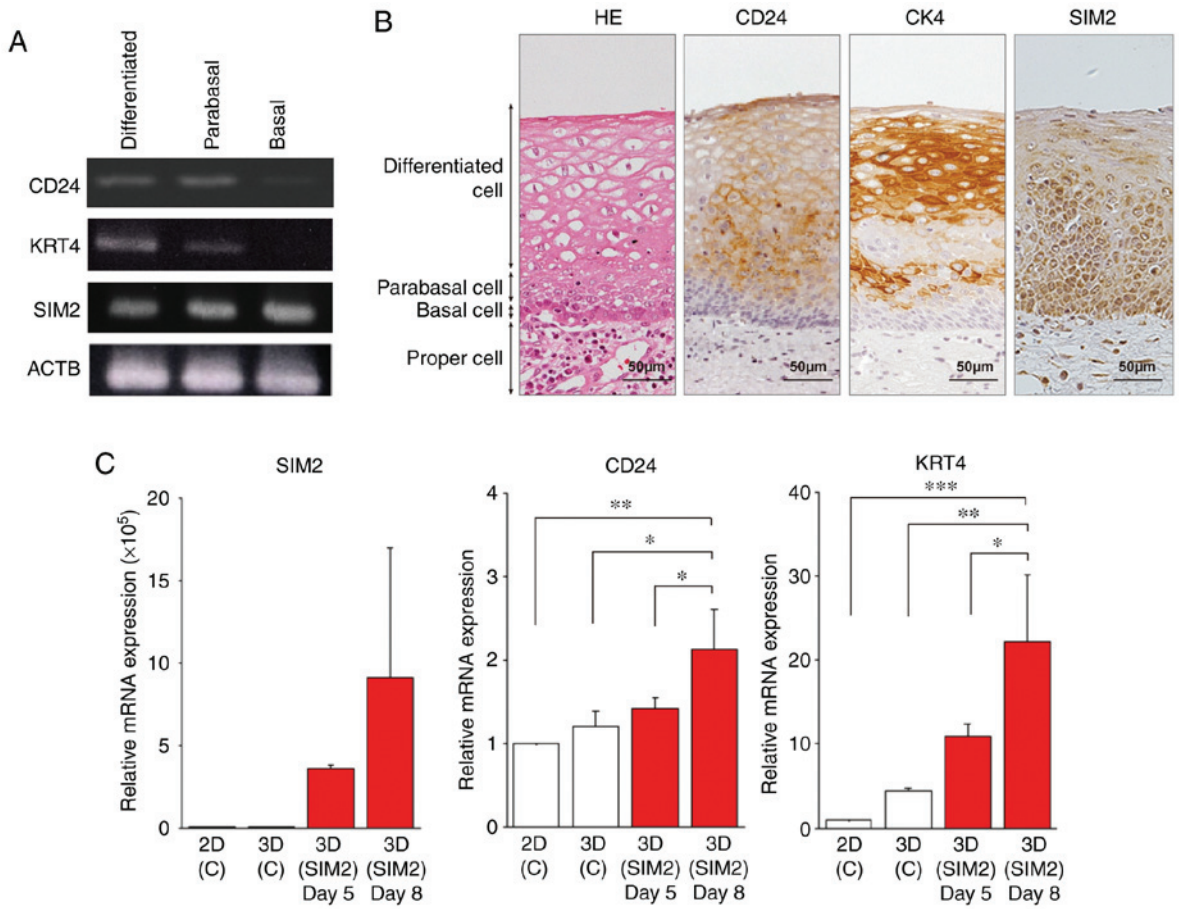

Figure 1. CD24 and CK2, which is encoded by *KRT4*, are differentiation markers regulated by SIM2. (A) Semi-quantitative RT-PCR of *CD24* and *KRT4* in three layers (differentiated, parabasal and basal cell layers) of the normal esophageal mucosa. (B) Immunohistochemical staining of CD24, CK4 and SIM2 in the normal esophageal mucosa; representative images are indicated. (C) RT-PCR of *SIM2*, *CD24* and *KRT4* in 3D-cultured T.Tn cells 5 or 8 days after empty vector or *SIM2* transfection (n=3, mean ± SE). *P<0.05, **P<0.01 and ***P<0.001. ACTB, β-actin; CK4, cytokeratin 4; HE, hematoxylin and eosin; KRT4, keratin 4; RT-PCR, reverse transcription-PCR; SIM2, SIM bHLH transcription factor 2.

exhibited low expression of both CD24 and CK4. Patients with high CD24 + CK4 expression survived longer than patients with low CD24 + CK4 expression (Fig. 3B).

Multivariate Cox regression analysis in 81 patients with ESCC revealed that high CD24 or CK4 expression was an independent favorable prognostic factor in response to definitive CRT for RFS (CD24: HR, 0.451; 95% CI, 0.204-0.997; P=0.049 and CK4: HR, 0.289; 95% CI, 0.009-0.960; P=0.043) and OS (CD24: HR, 0.281; 95% CI, 0.108-0.732; P=0.009 and CK4: HR, 0.119; 95% CI, 0.016-0.894; P=0.039) (Table II). Tumor differentiation type (tissue type) of biopsy specimens was also revealed to be an independent favorable prognostic factor for OS, but not for DFS, in response to definitive CRT (Table II). In accordance with CD24 and CK4 being differentiation markers (Fig. 1), ESCC samples with high CD24 or CK4 expression, particularly CD24, divided preferentially into well or moderately differentiated cancer (Table SII).

CD24 and CK4 are predictive biomarkers for definitive CRT and surgery. Based on the clinicopathological characteristics of the patients (Table SI), 81 patients with ESCC undergoing CRT were compared with 63 patients with ESCC undergoing surgery. Kaplan-Meier analyses revealed that when CD24 was highly expressed, there was no significant difference in the RFS and OS of 26 patients with ESCC undergoing definitive CRT compared with the 33 patients with ESCC undergoing surgery. Conversely, when CD24 was lowly expressed, there was a significant difference between the RFS and OS of 55 patients with ESCC undergoing definitive CRT and those of 30 patients with ESCC undergoing surgery (Fig. 4A). Although there were more patients with CK4 high expression in the CRT group, when CK4 was highly expressed, there was no significant difference in the RFS and OS of patients undergoing definitive CRT compared with those undergoing surgery (Fig. 4B). Conversely, when CK4 was lowly expressed, there was a significant difference in the RFS and OS of patients undergoing CRT compared with those undergoing surgery (Fig. 4B). As shown in Tables III and IV, multivariate Cox regression analysis in patients with ESCC and low CD24 or CK4 expression indicated that there was a significant difference between patients undergoing definitive CRT and those undergoing surgery in RFS (low CD24 HR, 2.28; 95% CI, 1.182-4.397; P=0.014 and low CK4: HR, 2.142; 95% CI, 1.274-3.599; P=0.004) and OS (low CD24: HR, 3.781; 95% CI, 1.518-9.416; P=0.004 and low CK4: HR, 2.407; 95% CI, 1.317-4.399; P=0.004). However, in patients with ESCC and high CD24 or CK4, there was no significant difference between RFS and OS between CRT and surgery (data not shown). Taken together, in cases with low

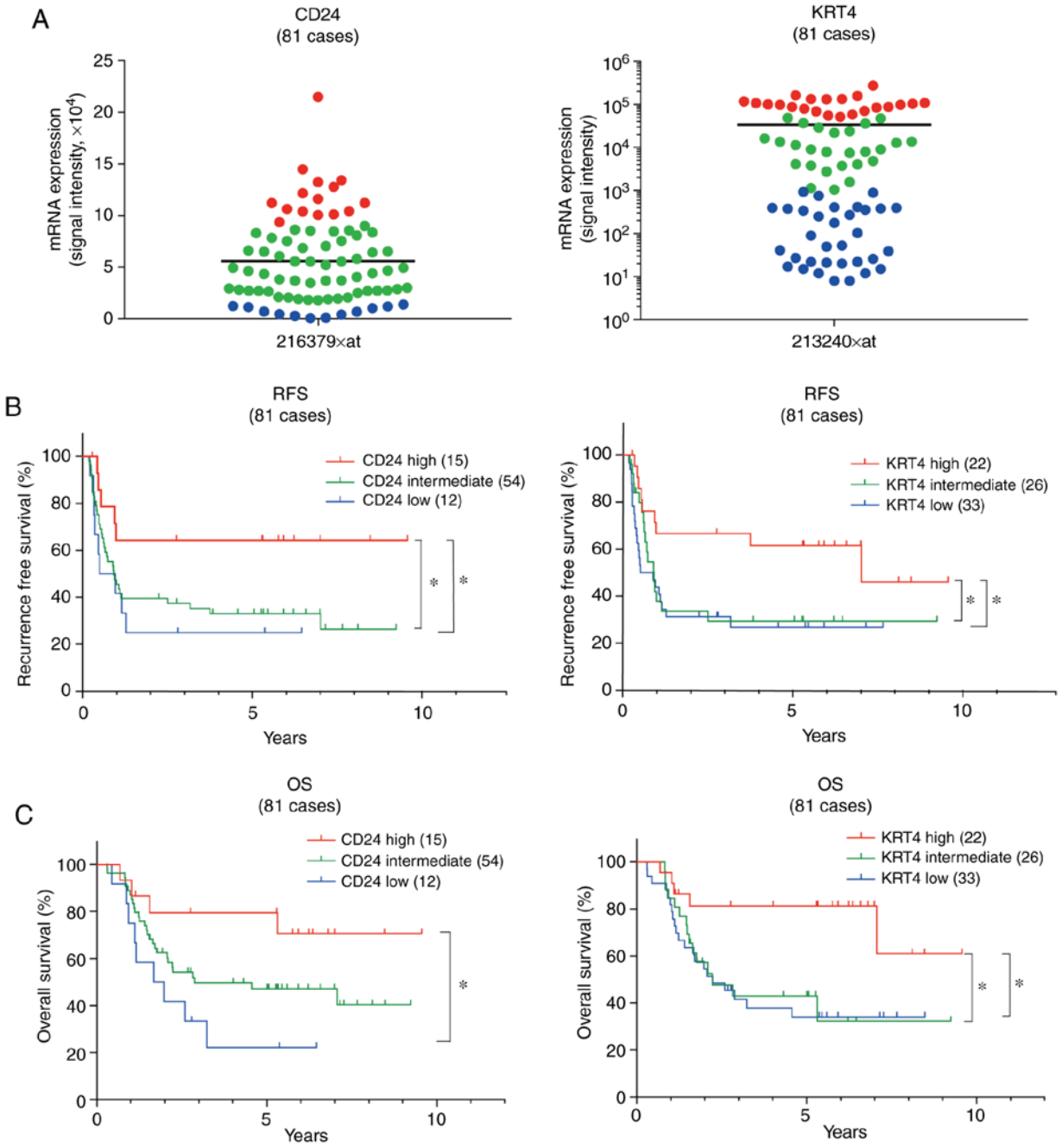

Figure 2. Patients with ESCC and high *CD24* and *KRT4* mRNA expression exhibit a favorable prognosis with definitive CRT. (A) Using our microarray data (GSE69925), *CD24* and *KRT4* mRNA expression was examined in 81 biopsy specimens prior to definitive CRT. A total of 15 of the 81 cases (18.5%) were classified into a high *CD24* expression group (red, expression was higher than the mean ± SD). Similarly, 22 of the 81 cases (27%) were classified into a high *KRT4* expression group (red, expression was >50,000 in signal intensity). Bar indicates the mean. (B and C) Kaplan-Meier analysis revealed that RFS and OS of the high *CD24* and *KRT4* expression groups were significantly longer than those of the low *CD24* and *KRT4* expression groups (blue, CD24 expression was lower than the mean-SD; KRT4, expression was <1,000 in signal intensity). *P<0.05. KRT4, keratin 4; OS, overall survival; RFS, recurrence-free survival.

CD24 or CK4, surgery was revealed to be a good therapeutic modality compared with definitive CRT.

CD24 is associated with radiosensitivity through superoxide dismutase 2 (SOD2) suppression, but not chemosensitivity in ESCC cells. In the present study, microarray and IHC analyses of biopsy specimens from 81 patients with ESCC prior to definitive CRT revealed that if CD24 mRNA or protein was highly expressed, RFS and OS were better (Figs. 2 and 3). Furthermore, we recently reported that SIM2 expression was associated with a favorable prognosis of patients with ESCC undergoing definitive CRT, and that SIM2 was involved in chemosensitivity through suppression of numerous DNA repair genes (X-ray repair cross complementing 5, BRCA1 DNA repair-associated, FA complementation group D2 and BRCA1-asssociated RING domain 1) and radiosensitivity through antioxidant gene (*SOD2*) suppression (13). These findings indicated that *CD24* may be directly involved in chemosensitivity and/or radiosensitivity. RT-qPCR was carried out using two *CD24* siRNAs (CD24-s2615 and CD24-s2616), and a decrease in *CD24*

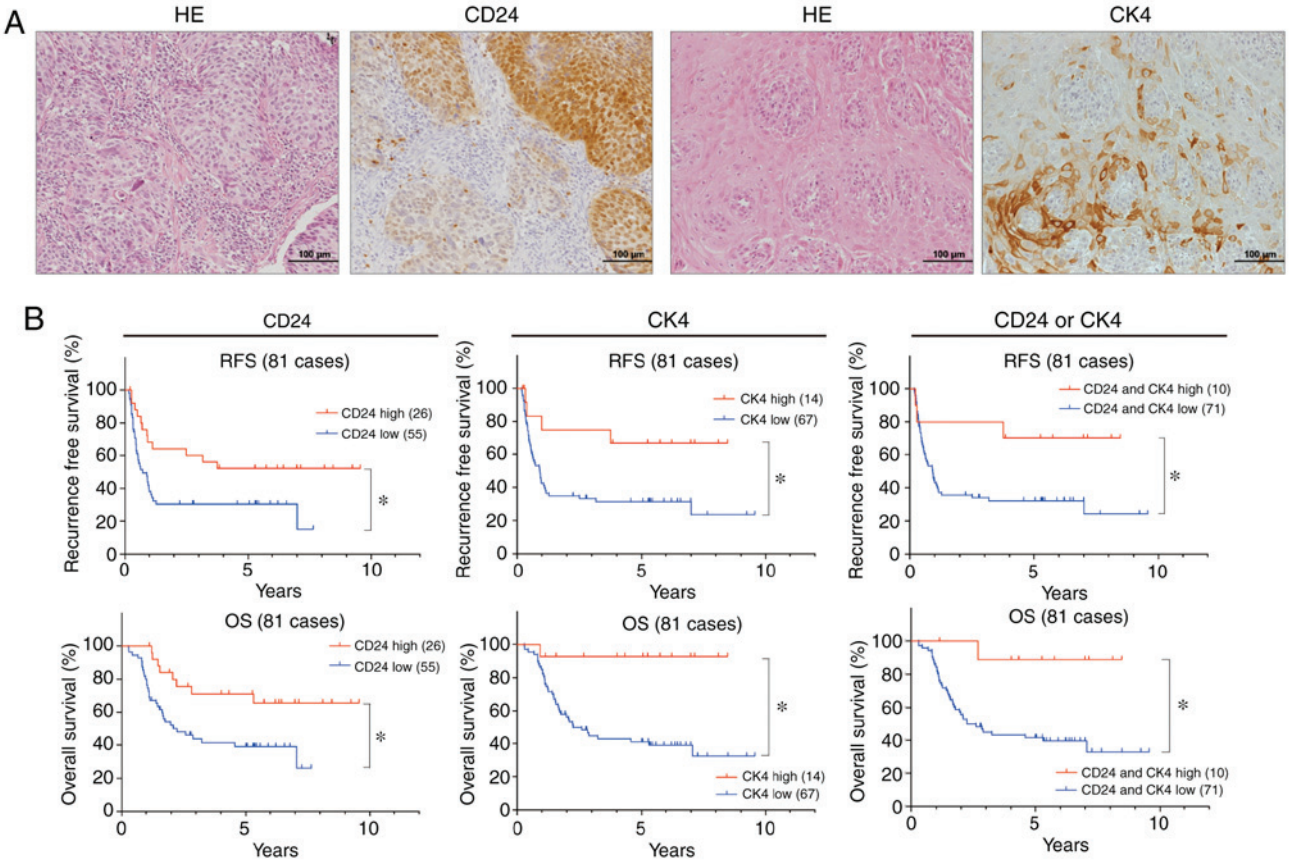

Figure 3. Patients with ESCC and high CD24 and CK4 protein expression exhibit a favorable prognosis with definitive CRT. (A) Representative immunohistochemical staining of CD24 and CK4. (B) High CD24 expression (>20% positive) was detected in 26 out of 81 patients (32%), whereas high CK4 expression (>10% positive) was detected in 14 out of 81 patients (17%). RFS and OS of patients with high CD24 or CK4 expression were significantly higher than those of patients with low CD24 or CK4 expression. In addition, 10 patients with high CD24 + CK4 expression survived longer than the 71 patients with low CD24 + CK4 expression. *P<0.05. CK4, cytokeratin 4; HE, hematoxylin and eosin; OS, overall survival; RFS, recurrence-free survival.

mRNA expression was confirmed (Fig. 5A). Accordingly, CD24 protein expression was also decreased by *CD24* siRNA (Fig. 5A). To examine the hypothesis that CD24 is involved in CRT sensitivity, control siRNA-, *CD24* siRNA (s2615)- and *CD24* siRNA (s2616)-transfected T.Tn cells were treated with CDDP, which is used in the standard chemotherapy regimen of ESCC, for 3 days in a 3D culture. The viable ratio of *CD24* siRNA (s2615)- or *CD24* siRNA (s2616)-transfected T.Tn cells was not significantly decreased compared with control siRNA-transfected T.Tn cells (Fig. 5B), suggesting that *CD24* was not involved in chemosensitivity. However, *CD24* siRNA (s2615)- or *CD24* siRNA (s2616)-transfected T.Tn cells exhibited increased *SOD2* mRNA expression compared with in the control siRNA-transfected T.Tn cells (Fig. 5C). In addition, *CD24* siRNAs were transfected into T.Tn cells and cell viability was investigated after H_2O_2 treatment. *CD24* siRNA (s2615)- or *CD24* siRNA (s2616)-transfected T.Tn cells exhibited significantly increased viability following H_2O_2 treatment compared with in the control siRNA-transfected T.Tn cells (Fig. 5D). These findings indicated that CD24 may be involved in radiosensitivity through *SOD2* suppression, but not in chemosensitivity (Fig. 6).

Discussion

Although definitive CRT improves the prognosis of patients with ESCC and is an important modality, ~40% of patients exhibit persistent disease or experience recurrence, resulting in poor long-term survival (2). Therefore, predictive biomarkers are needed to select patients who are potentially curable with definitive CRT. Since preoperative treatment is increasing for patients with solid tumors, biopsy specimens of such patients are the only material available that may be used to predict the effect of neoadjuvant therapy. Great efforts have been made to identify such predictive biomarkers by numerous researchers; however, few studies exist that have identifed biomarkers for definitive CRT using biopsy specimens from patients with ESCC (4,33). In this study, it was demonstrated that CD24 and CK4 have great potential to be independent predictive biomarkers for such patients. Our recent study reported that SIM2 in ESCC was a key transcription factor involved in tumor cell differentiation and was associated with a good response to CRT (13). This study revealed that *CD24* and *KRT4*, which encodes CK4, were differentiation markers, which were upregulated by *SIM2*. Therefore, *CD24* and *KRT4* may be downstream differentiation markers of *SIM2*, and similar to *SIM2*, they may serve a role in CRT sensitivity.

Kaplan-Meier analyses revealed that RFS and OS in the high *CD24* and *KRT4* mRNA expression groups were significantly longer than those in the low *CD24* and *KRT4* mRNA expression groups. In addition, immunohistochemical analyses were conducted, and the power of CD24 and CK4 for predicting patients with ESCC and a favorable prognosis

Table II. Multivariate analysis of RFS and OS in patients with ESCC undergoing definitive CRT.

		RFS			OS		
Variable	n (%)	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age							
<60 years	21 (25.9)	Reference	0.468-1.976	0.914	Reference	0.776-2.958	0.528
≥60 years	60 (74.1)	0.961		0.62	0.764		
Sex							
Male	74 (91.3)	Reference	0.474-3.470	0.624	Reference	0.585-5.368	0.311
Female	4 (8.7)	1.283			1.772		
Macroscopic type							
Types 1 and 2	50 (61.7)	Reference	0.572-1.934	0.87	Reference	0.776-2.958	0.224
Type 3	31 (38.3)	1.052			1.515		
Tissue type							
W/D and M/D	68 (84.0)	Reference	0.658-3.518	0.327	Reference	1.045-7.294	0.041[a]
P/D	13 (16.0)	1.521			2.76		
Location							
Ut and Mt	45 (55.6)	Reference	0.420-1.441	0.425	Reference	0.416-1.555	0.518
Lt	36 (44.4)	0.778			0.805		
Circumference							
<3/4	45 (55.6)	Reference	0.822-2.761	0.185	Reference	0.975-3.618	0.06
≥3/4	36 (44.4)	1.507			1.878		
c T factor							
T2	16 (19.8)	Reference	0.479-2.732	0.762	Reference	0.544-3.459	0.503
T3	65 (80.2)	1.144			1.372		
c N factor							
Absent	38 (46.9)	Reference	0.927-3.608	0.082	Reference	0.737-3.281	0.247
Present	43 (53.1)	1.828			1.555		
CD24							
Low	55 (67.9)	Reference	0.204-0.997	0.049[a]	Reference	0.108-0.732	0.009[a]
High	26 (32.1)	0.451			0.281		
CK4							
Low	67 (82.7)	Reference	0.009-0.960	0.043[a]	Reference	0.016-0.894	0.039[a]
High	14 (17.3)	0.289			0.119		

CK4, cytokeratin 4; OS, overall survival; c T, clinical Tumor; c N, clinical Node; Lt, lower thoracic; M/D, moderately differentiated; Mt, middle thoracic; P/D, poorly differentiated; RFS, recurrence-free survival; Ut, upper thoracic; W/D, well differentiated. [a]P<0.05.

in response to definitive CRT was evaluated. Multivariate Cox regression analyses revealed that high CD24 or CK4 expression was an independent favorable prognostic factor in patients undergoing definitive CRT. Notably, when CD24 or CK4 were highly expressed, there was no significant difference in RFS and OS between patients undergoing definitive CRT and those undergoing surgery. However, when CD24 or CK4 were lowly expressed, there was a significant difference in RFS and OS between patients undergoing definitive CRT and those undergoing surgery. Multivariate Cox regression analyses also indicated a significant difference in RFS and OS between patients undergoing definitive CRT and those undergoing surgery. During this study, discrepancies between mRNA and protein levels were detected in some individual cases. In high or low mRNA expression groups, these discrepancies are likely decreased if intermediate cases are removed from these groups, as one microarray analysis may have variability, particularly in cases with intermediate mRNA levels; therefore, cases were divided into three groups with regards to mRNA level (high, intermediate and low). In summary, for patients with ESCC and low CD24 or CK4 expression, it may be stated that surgery is preferable to definitive CRT. There were no significant changes in RFS and OS between patients undergoing definitive CRT and those undergoing surgery in the high CD24 or high CK4 groups; however, definitive CRT, which preserves organs, may be preferable for such patients.

In previous studies, CD24 overexpression has been reported to be markedly associated with a more aggressive

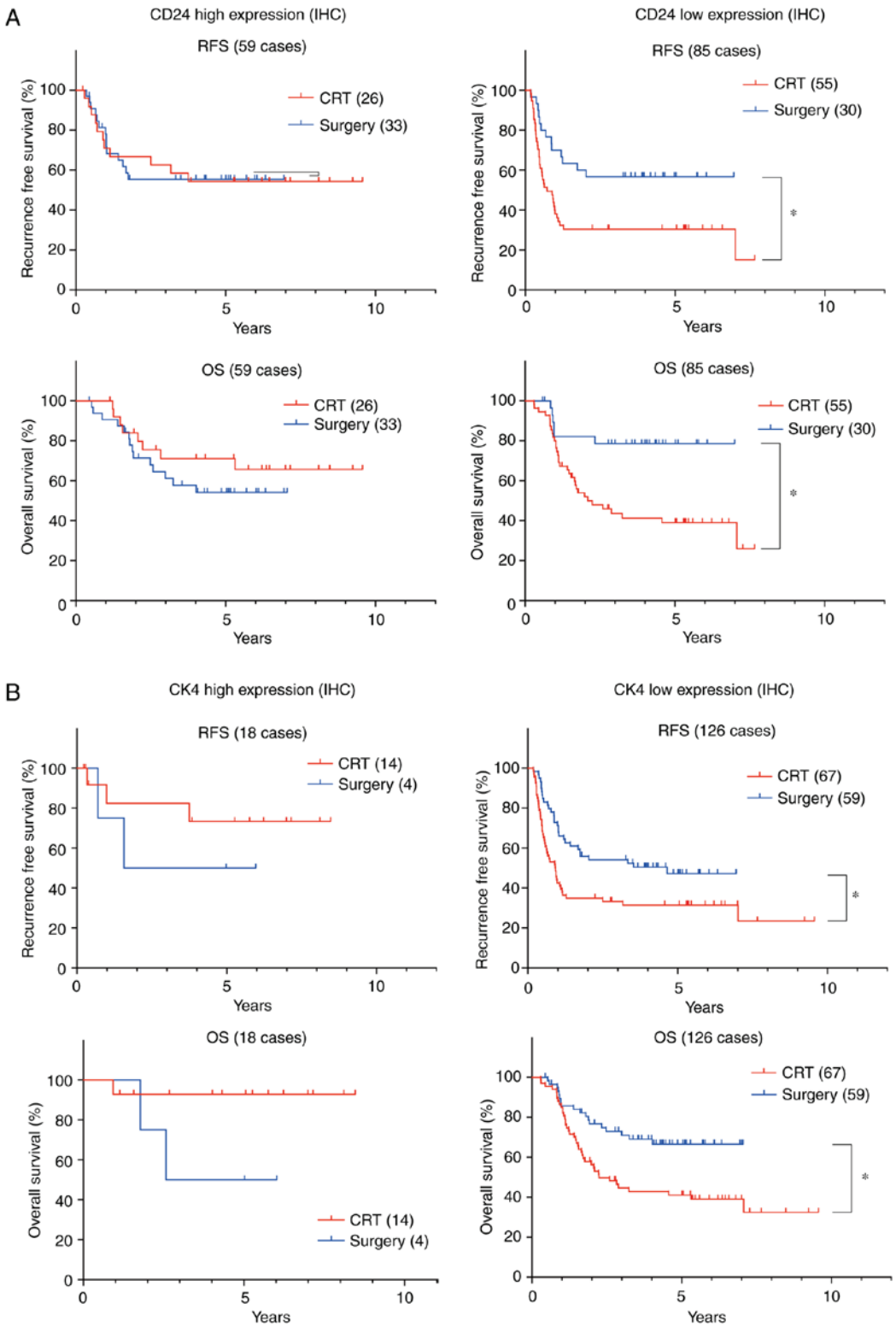

Figure 4. CD24 and CK4 are predictive biomarkers for definitive CRT and surgery. (A) Based on CD24 and CK4 protein expression, prognosis was compared between 81 patients with ESCC undergoing CRT and 63 patients with ESCC undergoing surgery. In patients with high CD24 expression, there was no significant difference in RFS and OS between 26 patients undergoing definitive CRT and 33 patients undergoing surgery, whereas in patients with low CD24 expression, there was a significant difference in RFS and OS between 55 patients undergoing definitive CRT and 30 patients undergoing surgery. (B) Similarly, in patients with high CK4 expression, there was no significant difference in RFS and OS between 14 patients undergoing definitive CRT and four patients undergoing surgery, whereas in patients with low CK4 expression, there was a significant difference in RFS and OS between 67 patients undergoing definitive CRT and 59 patients undergoing surgery. *P<0.05. CK4, cytokeratin; CRT, chemoradiotherapy; IHC, immunohistochemistry; OS, overall survival; RFS, recurrence-free survival.

course of disease (15-18). CD24 may have a role in breast cancer metastasis (19-21) and has been identified as a significant poor prognostic factor (34). In ovarian cancer, CD24 is a key molecule in epithelial-mesenchymal transition

Table III. Multivariate analysis of RFS and OS in patients with low CD24 expression.

		RFS			OS		
Variable	n (%)	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age							
<60 years	18 (21.2)	Reference	0.559-2.573	0.64	Reference	0.453-2.582	0.86
≥60 years	67 (78.8)	1.2			1.082		
Sex							
Male	75 (88.2)	Reference	0.421-2.822	0.86	Reference	0.723-5.161	0.189
Female	10 (11.8)	1.089			1.931		
Macroscopic types							
Types 1 and 2	51 (60.0)	Reference	0.588-1.867	0.874	Reference	0.930-3.474	0.081
Type 3	34 (40.0)	1.048			1.798		
Tissue type							
W/D and M/D	75 (88.2)	Reference	0.488-2.857	0.721	Reference	0.924-5.869	0.073
P/D	10 (11.8)	1.181			2.328		
Location							
Ut and Mt	78 (91.8)	Reference	0.707-2.224	0.439	Reference	0.666-2.493	0.452
Lt	7 (8.2)	1.254			1.288		
Circumference							
<3/4	49 (57.6)	Reference	0.858-2.826	0.145	Reference	0.995-4.040	0.052
≥3/4	36 (42.4)	1.557			2.005		
c T factor							
T2	14 (16.5)	Reference	0.759-4.734	0.171	Reference	0.453-3.356	0.682
T3	71 (83.5)	1.896			1.233		
c N factor							
Absent	41 (48.2)	Reference	0.601-2.024	0.751	Reference	0.560-2.274	0.736
Present	44 (51.8)	1.103			1.128		
Treatment							
Surgery	30 (35.3)	Reference	1.182-4.397	0.014[a]	Reference	1.518-9.416	0.004[a]
CRT	55 (64.7)	2.28			3.781		

CRT, chemoradiotherapy; OS, overall survival; c T, clinical Tumor; c N, clinical Node; Lt, lower thoracic; M/D, moderately differentiated; Mt, middle thoracic; P/D, poorly differentiated; RFS, recurrence-free survival; Ut, upper thoracic; W/D, well differentiated. [a]P<0.05.

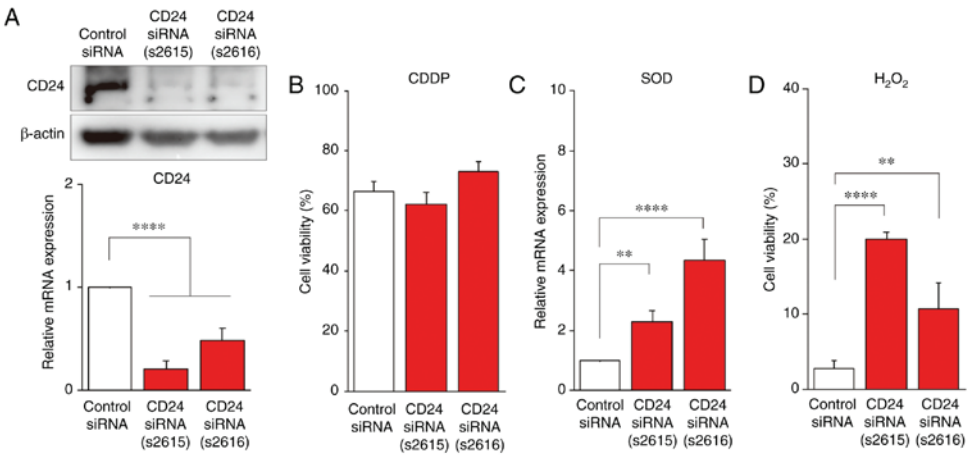

Figure 5. CD24 is involved in radiosensitivity through *SOD2* suppression, but not in chemosensitivity of ESCC cells. (A) CD24 protein and *CD24* mRNA expression levels were decreased 3 days post-transfection with two *CD24* siRNAs (CD24-s2615 and CD24-s2616) (n=3, mean ± SE). (B) Viability of T.Tn cells treated with CDDP 3 days post-transfection with *CD24* siRNA was not significantly decreased compared with control siRNA-transfected T.Tn cells. (C) *SOD2* mRNA expression was increased in *CD24* siRNA-transfected T.Tn cells compared with in control siRNA-transfected T.Tn cells (n=3, mean ± SE). (D) *CD24* siRNA-transfected T.Tn cells exhibited significantly increased cell viability 24 h after treatment with H_2O_2 compared with in control siRNA-transfected T.Tn cells (n=3, mean ± SE). **P<0.01, ***P<0.001 and ****P<0.0001. CDDP, cisplatin; SOD2, superoxide dismutase 2.

Table IV. Multivariate analysis of RFS and OS in patients with low CK4 expression.

		RFS			OS		
Variable	n (%)	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age							
<60 years	30 (23.8)	Reference	0.689-2.236	0.472	Reference	0.604-2.218	0.659
≥60 years	96 (76.2)	1.241			1.158		
Sex							
Male	111 (88.1)	Reference	0.639-2.698	0.459	Reference	0.938-4.367	0.072
Female	15 (11.9)	1.313			2.023		
Macroscopic types							
Types 1 and 2	78 (61.9)	Reference	0.626-1.688	0.913	Reference	0.371-1.100	0.106
Type 3	48 (38.1)	1.028			0.639		
Tissue type							
W/D and M/D	108 (85.7)	Reference	0.508-2.086	0.935	Reference	0.380-1.695	0.565
P/D	18 (14.3)	1.03			0.803		
Location							
Ut and Mt	69 (54.8)	Reference	0.419-1.126	0.137	Reference	0.483-1.419	0.492
Lt	57 (45.2)	0.687			0.828		
Circumference							
<3/4	68 (54.0)	Reference	0.698-1.793	0.64	Reference	0.918-2.609	0.101
≥3/4	58 (46.0)	1.119			1.548		
c T factor							
T2	26 (20.6)	Reference	1.139-4.838	0.021[a]	Reference	0.711-3.173	0.286
T3	100 (79.4)	2.347			1.502		
c N factor							
Absent	67 (53.2)	Reference	0.748-1.967	0.434	Reference	0.557-1.621	0.851
Present	59 (46.8)	1.213			0.95		
Treatment							
Surgery	59 (46.8)	Reference	1.274-3.599	0.004[a]	Reference	1.317-4.399	0.004[a]
CRT	67 (53.2)	2.142			2.407		

CRT, chemoradiotherapy; OS, overall survival; c T, clinical Tumor; c N, clinical Node; Lt, lower thoracic; M/D, moderately differentiated; Mt, middle thoracic; P/D, poorly differentiated; RFS, recurrence-free survival; Ut, upper thoracic; W/D, well differentiated. [a]P<0.05.

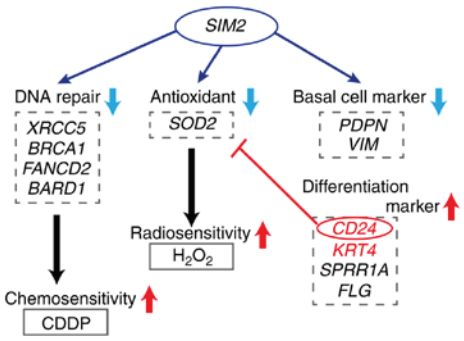

Figure 6. Schematic summary of the possible roles of SIM2 in definitive CRT for ESCC. CD24 may be involved in radiosensitivity through SOD2 suppression, but not chemosensitivity.

(EMT) (35). Furthermore, downregulation of CD24 has been reported to suppress bone metastasis of lung cancer cells (36). However, the role of CD24 in ESCC remains to be determined.

Our recent studies reported that transfection with *SIM2* reduced the podoplanin (PDPN)-positive basal cell ratio and improved sensitivity to CDDP (12,13). Knockdown of PDPN has been reported to reduce resistance to CDDP (37). In the present study, in response to CDDP, the number of viable *CD24* siRNA-transfected cells was not significantly decreased compared with the control cells, suggesting that CD24 was not involved in chemosensitivity. SOD2 is known to efficiently catalyze the dismutation of reactive oxygen species (38), which are induced by irradiation. This study demonstrated that CD24 may suppress *SOD2* expression and thus reduce resistance to H_2O_2. These data indicated that CD24 may be involved in radiosensitivity through SOD2 suppression, but not in chemosensitivity (Fig. 6).

Transforming growth factor (TGF)-β is a major inducer of EMT during embryonic development, as well as the pathogenesis

of fibrotic disorders and cancer progression (39-41). In ovarian cancer, CD24 and EMT regulators have been reported to be induced by TGF-β (35). This study investigated whether TGF-β stimulated the expression of EMT regulator genes (*TWIST1*, *TWIST2* and *SNAI2*), mesenchymal cell marker genes (*CDH2* and *VIM*) and *CD24*. As shown in Fig. S2, TGF-β upregulated *CDH2*, *VIM* and *SNAI2*, but downregulated *CD24*, *TWIST1* and *TWIST2* in T.Tn cells, suggesting that CD24 was not involved in TGF-β-mediated EMT in ESCC.

In conclusion, the results of the present study may foster development of the predictive biomarkers CD24 and CK4 for selection of the best therapeutic modality, including definitive CRT, in ESCC. It was hypothesized that IHC of CD24 and CK4 may be useful for patient stratification; however, biopsy samples are often too small (2x2 mm) to show a significant difference. For clinical use, the cut-off values should be determined by future extensive immunohistochemical analyses using several sections from multi-institutional cohorts.

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Availability of data and materials

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

Authors' contributions

KT, HS and TY contributed to the study conception and design. RK, MK and HS performed the microarray data analyses. KT, SF, MT and TY performed and evaluated IHC. RK, KT, FC and HS performed the cell line experiments. KT, TK, HD, KM, MM and TY analyzed the patient data. KT, SF, RK, FC and HS drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants in this study. All procedures were approved by the responsible committee on human experimentation at National Cancer Center East (approval no. 16-97), and were conducted in accordance with the Helsinki Declaration.

Patient consent for publication

Patients provided informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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