CD44v3$^+$/CD24$^-$ cells possess cancer stem cell-like properties in human oral squamous cell carcinoma

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Abstract. Cancer stem cells (CSCs) or cancer stem cell-like cells (CSC-LCs) are a minority population of cells that relate to tumor progression, metastasis and drug resistance. To identify CSC-LCs in oral squamous cell carcinoma (OSCC), we used two OSCC cell lines, SAS and OSC20, and cell surface markers, CD44v3 and CD24. In addition, we examined CD44v3 and CD24 expression immunohistochemically and evaluated the relationship between the expression and clinicopathological parameters in 50 OSCC tissues. In SAS and OSC20, CD44v3$^+$/CD24$^-$ cells showed a higher sphere forming ability than the other fractions, i.e., CD44v3$^+$/CD24$^+$, CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$ cells. The proportion of CD44v3$^+$/CD24$^-$ cells in SAS and OSC20 was 10.7 and 24.1%, respectively. Regarding SAS, CD44v3$^+$/CD24$^-$ cells also showed a higher drug resistance for CDDP, 5-FU and cetuximab and expressed higher mRNA levels of CSC property-related genes than the other cell fractions. The tumorigenicity of CD44v3$^+$/CD24$^-$ cells was not significantly different from the other fractions in SAS. An immunohistochemical study revealed a significant correlation between CD44v3 expression in the invasive portion and lymph node metastasis. Kaplan Meier analysis revealed cases with CD44v3 expression in the invasive portion tended to show poor overall survival (OS) compared with those without CD44v3, and there was a significant difference in OS between CD44v3$^+$/CD24$^-$ and CD44v3$^-$/CD24$^-$ immunophenotypes in the invasive portion. In conclusion, the results suggest that the CD44v3$^+$/CD24$^-$ cell population displays CSC-LC properties in a human OSCC cell line. Additionally, we present evidence that CD44v3 immunoexpression and CD44v3$^+$/CD24$^-$ immunophenotypes could give prognostic information associated with unfavorable clinical outcomes.

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Introduction

Recently, a number of studies identified cell surface markers, such as CD44v3 and CD24, as CSC markers (1-7). Oral squamous cell carcinoma (OSCC) is the eighth most prevalent cancer worldwide and shows high morbidity and poor survival rates (8). Despite advances in therapeutic procedures and various combinations of chemotherapeutic agents that have improved quality of life, mortality from this disease remains high because of the development of distant metastasis and the emergence of local and regional recurrences. Such treatment failure may be due to a small population of cells [cancer stem cells (CSCs)] that are responsible for tumorigenesis and contribute to resistance to conventional therapy, such as chemotherapy and radiotherapy. Therefore, identification of CSCs or cancer stem cell-like cells (CSC-LCs) may lead to the development of effective treatment. Many reports have verified the existence of CSCs in various solid neoplasms (7,9-12). With regard to OSCC, Prince *et al* (13) identified a population of CD44 positive tumor initiating cells in OSCC. Chen *et al* (14) reported that OSCC harbored potential CSC characterized by ALDH1.

CD44v3 is an alternative splicing form variant of CD44, which is a multifunctional transmembrane glycoprotein expressed in many types of cancer. With regard to OSCC, several studies have reported that CD44v3 is associated with drug resistance and unfavorable clinical outcomes (15,16).

CD24 is a 27-amino-acid single-chain protein that is O- and N-glycosylated and is bound to the extracellular matrix (17) and the extracellular membrane by a glycosylphosphatidylinositol anchor (18). Although several studies have reported that CD24 is associated with invasion, metastasis and tumor differentiation (19,20), whether CD24 expression is upregulated or downregulated with tumor invasion remains unclear. CD24 has also been studied in combination with CD44. Several studies have reported that CD44$^+$/CD24$^-$ cells showed CSC properties in breast and prostate cancer (4-6). On the other hand, several studies have reported that CD44$^+$/CD24$^+$ was the CSC phenotype of pancreatic and colorectal cancer (7,21). With regard to OSCC, a few reports have shown that CD44$^+$/CD24$^-$ may be the CSC phenotype (22,23).

In the present study, we focused on CD44v3 and CD24 and examined whether these markers have CSC properties by using two human OSCC cell lines and 50 human OSCC tissues.

Materials and methods

Cell lines and media. Two OSCC cell lines, SAS and OSC20, both derived from primary lesions of a patient with OSCC, were used in the experiment. SAS was purchased from the Health Science Research Resources Bank. OSC20 was donated by the Research Center for Innovative Cancer Therapy, Molecular Targeting Therapeutics Division, Kurume University, School of Medicine. SAS was grown in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku Co., Ltd., Tokyo, Japan) and Ham's F12 medium supplemented with heat-inactivated (56˚C, 30 min) 5% fetal bovine serum (FBS; Bioserum, Victoria, Australia), 100 U/ml, penicillin and 100 μg streptomycin (Gibco-BRL/Life Technologies Inc., Gaitherburg, MD, USA). OSC20 was grown in Eagle's minimum essential medium (EMEM; Gibco, BRL/Life Technologies Inc.) with 5% FBS. Cells were cultured in an atmosphere of 5% CO_2 in air at 37˚C.

Flow cytometric analysis and separation. SAS and OSC20 cells with 80% confluence were washed once with phosphate-buffered saline (PBS), detached with accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA), suspended at $1x10^6$ cells/ml in PBS supplemented with 2% FBS, incubated with 10 μg/ ml human IgG (R&D Systems, Inc., Minneapolis, MN, USA) for 15 min at room temperature, and then incubated with allophycocyanin (APC)-conjugated mouse anti-human CD44v3 (cat. no. FAB5088A; R&D Systems) combined with phycoerythrin (PE)-conjugated mouse anti-human CD24 (cat. no. 555428; BD Biosciences, San Jose, CA, USA) at 4˚C for 45 min. Samples were washed, centrifuged at 500 x g for 3 min, resuspended in 2 ml cold PBS supplemented with 2% FBS, then 1 μg/ml propidium iodide (PI; BD Biosciences) was added and the cells were filtered through a 40-μm cell strainer (BD Biosciences). Analysis and separation were carried out with a FACSAria II (BD Biosciences).

Cell growth assay. A total of 2,500 cells of each of the four cell fractions, i.e., $CD44v3^+/CD24^-$, $CD44v3^+/CD24^+$, $CD44v3^-/CD24^-$ and $CD44v3^-/CD24^+$ cells isolated from two cell lines were plated in 96-well plates and cultured in a CO_2 incubator. The cells were harvested at 24, 48, 72 or 96 h and the proliferation was examined in colorimetric assays using 3-(4,5-dimethylthiazol-2yl-yl-)-2, 5-dimethyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon, Temecula, CA, USA) as described elsewhere (24).

Sphere forming assay. Four cell fractions isolated from two cell lines (5,000 cells/dish) were cultured in serum-free medium including 10 ng/ml epidermal growth factor (EGF; Sanko Junyaku Co., Ltd., Tokyo, Japan) and 20 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, NJ, USA) using ultra-low attachment 6-well plates (Corning Inc., Corning, NY, USA) for 1 week. Sphere formation was assessed by counting the number of spheres (>3 cells) under a microscope (x200).

Drug treatment assay. The four isolated cell fractions sorted from two cell lines were plated at 2,500 cells/well in 96-well plates, and the effect of CDDP (1 or 5 μM) (Nihon Kayaku, Tokyo, Japan), 5-fluorouracil (5-FU) (10 or 100 μM) (Kyowa Hakko, Tokyo, Japan), and cetuximab (100 or 1,000 nM) (Merck Serono Co., Ltd., Tokyo, Japan) was examined. Drug resistance was determined after treatment for 96 h by MTT assay.

Tumorigenicity assay. Various numbers of cells ($1x10^3$, $1x10^4$, or $1x10^5$) were injected subcutaneously into 4-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (n=5 in each group). Tumorigenic capacity was judged 8 weeks after injection. The animal procedures were approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine.

cDNA preparation and quantitative real-time RT-PCR for gene expression assay. After the four fractions were isolated, total RNA was extracted using an RNAqueous Micro kit (Life Technologies, Carisbad, CA, USA), and complementary DNA (cDNA) was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR (RT-qPCR) was performed to examine the expression of CSC-LC property-related genes [e.g., ABC transporter genes (ABCB1 and ABCG2), anti-apoptosis genes (BCL2 and CFLAR), self-replication genes (Oct4 and Nanog) and hypoxia-related genes [hypoxia inducible factor 1α (HIF1α)] with ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Gene expression assays and primer and probe mixes were used for ABCB1, ABCG2, ALDH1A1, BCL2, CFLAR, Oct4, Nanog, HIF1α, and β-actin [assay IDs (Hs 00184500_m1, Hs01053790_m1, Hs00946916_m1, Hs00608023_m1, Hs00153439_m1, Hs03666771, Hs04260366, Hs00153153_m1, and Hs99999903_m1, respectively; Applied Biosystems)], and thermal conditions were as follows: initial incubation at 95˚C for 10 min, then 40 cycles alternating in turn with 95˚C for 10 sec, 60˚C for 20 sec, and 72˚C for 15 sec, and then maintained at 72˚C for 10 min. Comparative gene expression analysis was performed using the $2^{(-\Delta\Delta Cq)}$ method with normalization to the level of internal control gene, β-actin.

Tissue samples. OSCC tissue samples for immunohistochemistry were obtained from 50 patients who underwent surgical resection at Kurume University Hospital between 2007 and 2008. These specimens were fixed in 10% buffered formalin followed by paraffin embedment. None of the patients had previously received any treatments, including chemotherapy or radiotherapy. This cohort was composed of 33 men and 17 women aged from 48 to 87 years (median age, 70 years). The average observation time for overall survival was 52 months for patients still alive at the time of analysis, and ranged from 1 to 95 months.

Immunohistochemical staining. All tissues were immunohistochemically examined for expression of CD44v3 and CD24 using mouse anti-human CD44v3 monoclonal antibody (VFF-327v3, 1:50 dilution; Novocastra, New Castle upon Tyne, UK) and mouse anti-human CD24 monoclonal antibody

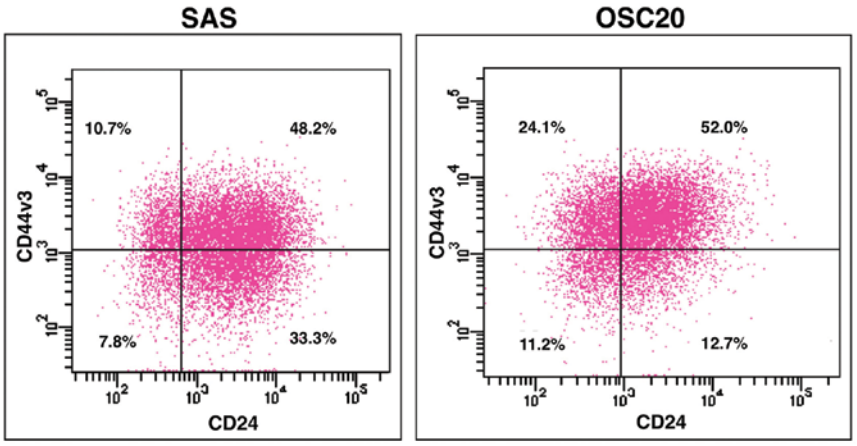

Figure 1. Expression of CD44v3/CD24 in SAS and OSC20. SAS and OSC20 are labeled with CD44v3 and CD24, and then analyzed by FCM. In SAS, the proportion of CD44v3$^+$/CD24$^-$, CD44v3$^+$/CD24$^+$, CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$ cells is 10.7, 48.2, 7.8 and 33.3%, respectively. Whereas, the proportion of CD44v3$^+$/CD24$^-$, CD44v3$^+$/CD24$^+$, CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$ cells is 24.1, 52.0, 11.2 and 12.7%, respectively.

(528807, 1:100 dilution; R&D Systems). BenchMark XT (Ventana Medical Systems, Inc., Tucson, AZ, USA) was used for immunostaining.

Evaluation of staining. All slides were evaluated by two of the authors (K.T. and J.A.). CD44v3 and CD24 expressions were evaluated according to staining intensity and staining area in the non-invasive portion and invasive portion within the tumor. The staining intensity was graded as 0, negative; 1, weakly positive; 2, moderately positive; or 3, strongly positive. The staining intensity in the normal epithelium was used as an internal control. The staining area was graded as 1, 0-20%; 2, 20-40%; 3, 40-60%; 4, 60-80%; and 5, 80-100%. The scores of staining intensity and area were calculated. The expression scores of CD44v3 and CD24 were compared between the non-invasive portion and invasive portion. The expression scores of CD44v3 and CD24 were categorized as either negative or positive. The median value of the expression score was used to separate the negative and positive groups. The relationship between isolated CD44v3 and isolated CD24 immunoexpression or CD44v3/CD24 immunophenotype and each clinicopathological feature of OSCC, such as T stage, nodal status, mode of invasion (25) and histological grade, was analyzed.

Statistical analysis. JMP software version 11.0 was used for all statistical analysis. Comparisons of cell growth assay, sphere forming assay, drug resistance assay, and quantitative real-time RT-PCR assay were normally distributed and assessed by the Shapiro-Wilk test, and then, a comparison of each *in vitro* assay except for the sphere forming assay was performed using one-way ANOVA with Dunnet post hoc comparisons. A comparison of the sphere forming assay was performed using one-way ANOVA with the Tukey post hoc comparisons. The expression score data of the invasive portion were compared with that of the non-invasive portion using a Mann-Whitney U test. Correlation of isolated CD44v3 and CD24 immunoexpression or the CD44v3/CD24 immunophenotypes with the clinicopathological parameters was assessed by standard Chi square tests or the Fisher's exact test. The overall survival rate was defined as the interval between the diagnosis and the date of death (uncensored data) or the date of the last available clinical information (censored data). Comparison and estimation of cumulative survival rates were performed using the Kaplan-Meier curves and the log rank test. All tests were two-sided and a value of P<0.05 was considered significant.

Results

Expression of CD44v3/CD24 in SAS and OSC20. In SAS, the proportion of CD44v3$^+$/CD24$^-$, CD44v3$^+$/CD24$^+$, CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$ cells was 10.7, 48.2, 7.8 and 33.3%, respectively. Whereas, the proportion of CD44v3$^+$/CD24$^-$, CD44v3$^+$/CD24$^+$, CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$ cells was 24.1, 52.0, 11.2 and 12.7%, respectively (Fig. 1).

Biological features of sorted cell fractions in SAS and OSC20 in vitro. CD44v3$^+$/CD24$^-$ cells in SAS showed a significantly higher proliferative ability than that of CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$ cells after culturing for 96 h (P<0.05), and, CD44v3$^+$/CD24$^-$ cells in OSC20 showed a significantly higher proliferative ability than that of CD44v3$^-$/CD24$^-$ cells (P<0.05) (Fig. 2A).

The sphere forming ability of CD44v3$^+$/CD24$^-$ cells in both SAS and OSC20 was significantly higher than the other fractions (P<0.05 or P<0.001). There was also a significant difference in sphere forming ability between the two fractions, for example, between CD44v3$^+$/CD24$^+$ and CD44v3$^-$/CD24$^-$, and between CD44v3$^+$/CD24$^+$ and CD44v3$^-$/CD24$^+$ cells (P<0.001) (Fig. 2B).

After 96-h treatment with CDDP, 5-FU, or cetuximab, the sensitivity to each drug was assessed with the MTT assay in SAS and OSC20. Cell growth was significantly suppressed in cells treated with CDDP, 5-FU and cetuximab, as compared with control cells in both SAS and OSC20 (Fig. 2C).

In SAS, CD44v3$^+$/CD24$^-$ cells had a significantly higher resistance in CDDP (1 μM) or 5-FU (10 or 100 μM) treatment than the other fractions (P<0.05 or P<0.001), and had

Table I. Tumorigenicity of sorted cell fractions in SAS.

	Injected cell number		
	$1x10^3$	$1x10^4$	$1x10^5$
$CD44v3^+/CD24^-$	0/5 (0%)	2/5 (40%)	2/5 (40%)
$CD44v3^+/CD24^+$	0/5 (0%)	2/5 (40%)	3/5 (60%)
$CD44v3^-/CD24^-$	0/5 (0%)	0/5 (0%)	1/5 (20%)
$CD44v3^-/CD24^+$	0/5 (0%)	0/5 (0%)	2/5 (40%)

a significantly higher resistance in CDDP (5 μM) or cetuximab (100 or 1,000 nM) treatment than $CD44v3^+/CD24^+$ and $CD44v3^-/CD24^+$ cells (P<0.001) (Fig. 2C).

In OSC20, $CD44v3^+/CD24^-$ cells had a significantly higher resistance in CDDP (1 or 5 μM) or 5-FU (100 μM) or cetuximab (100 nM) treatment than $CD44v3^+/CD24^+$ cells and/or $CD44v3^-/CD24^+$ cells (P<0.05, P<0.01 or P<0.001). There was no significant difference in sensitivity in 5-FU (10 μM) or cetuximab (1,000 nM) treatment among the cell fractions (Fig. 2C).

Analysis of CSC-LC property-related gene expression in sorted cell fractions in SAS and OSC20 by RT-qPCR. We performed RT-qPCR analysis to compare CSC-LC property related gene expression in $CD44v3^+/CD24^-$ cells and the other cell fractions in SAS and OSC20 cells. In SAS, $CD44v3^+/CD24^-$ cells showed significantly higher mRNA expression of transporter-related genes (ABCB1 and ABCG2), ALDH1A1, anti-apoptotic gene (BCL2), and self-replication genes (Oct-4, Nanog), than the other fractions (Fig. 3).

Tumorigenicity assays in vivo in sorted cell fractions in SAS. Injection of $1x10^3$ cells from all of the cell fractions produced no tumors in NOD/SCID mice. In contrast, two mice that received $1x10^4$ $CD44v3^+/CD24^-$ cells or $CD44v3^+/CD24^+$ cells developed tumors 8 weeks after the inoculation. In addition, injection of $1x10^5$ cells from all of the cell fractions produced tumors in NOD/SCID mice. The ratio of tumorigenicity among $CD44v3^+/CD24^-$ cell fractions and the other fractions in SAS was not significantly different (Table I).

Immunohistochemical findings and their relationship to clinicopathological features. CD44v3 and CD24 were predominantly expressed on the cell membrane and in the cytoplasm, respectively. A representative immunostaining

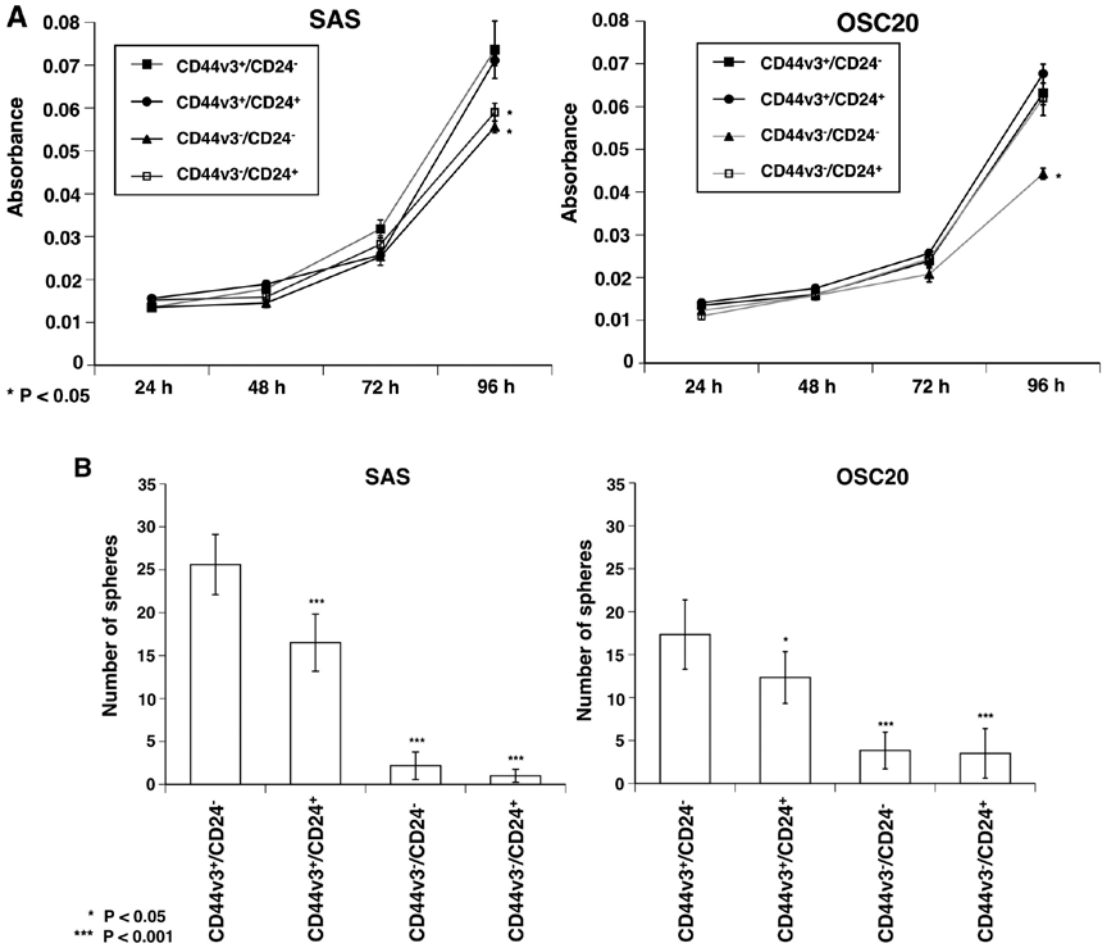

Figure 2. Biological features of sorted cell fractions in SAS and OSC20 *in vitro*. (A) $CD44v3^+/CD24^-$ cells in SAS show a higher proliferative ability than that of $CD44v3^-/CD24^-$ and $CD44v3^-/CD24^+$ cells. $CD44v3^+/CD24^-$ cells in OSC20 show a higher proliferative ability than that of $CD44v3^-/CD24^-$ cells (*P<0.05). (B) The sphere forming ability of $CD44v3^+/CD24^-$ cells in both SAS and OSC20 is significantly higher than in the other fractions (*P<0.05, ***P<0.001). The sphere forming ability of $CD44v3^+/CD24^+$ cells is also higher than that of $CD44v3^-/CD24^-$ and $CD44v3^-/CD24^+$ cells (***P<0.001).

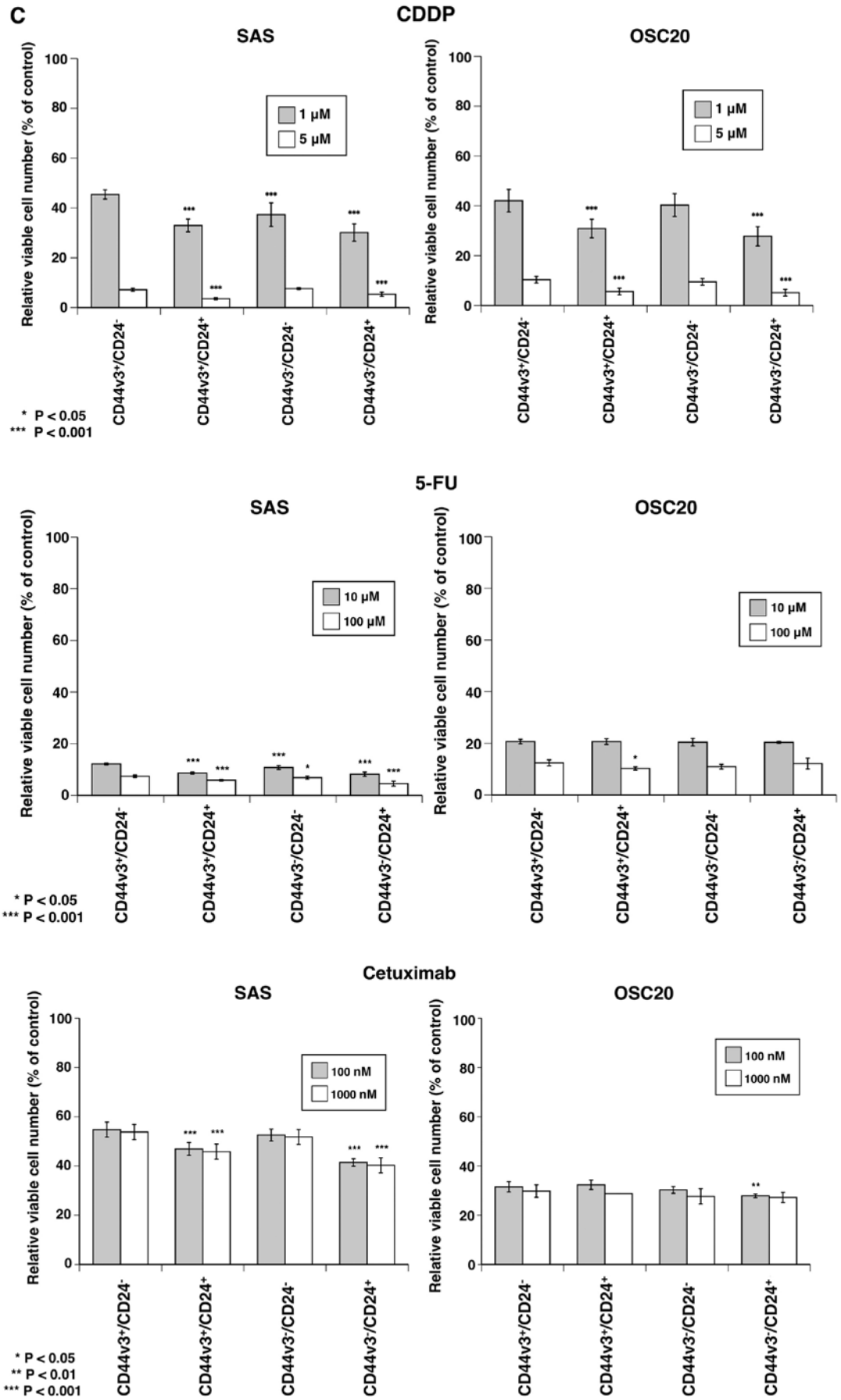

Figure 2. Continued. (C) In SAS, the viability of $CD44v3^+/CD24^-$ cells is significantly higher than the other fractions after treatment with 1 μM CDDP, or treatment with 10 or 100 μM of 5-FU and was significantly higher than that of $CD44v3^+/CD24^+$ or $CD44v3^-/CD24^+$ cells after treatment with 5 μM CDDP, or treatment with 100 or 1,000 nM cetuximab ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). In OSC20, the viability of $CD44v3^+/CD24^-$ cells is significantly higher than that of $CD44v3^+/CD24^+$ or $CD44v3^-/CD24^+$ cells after treatment with 1 or 5 μM CDDP ($^{***}P<0.001$).

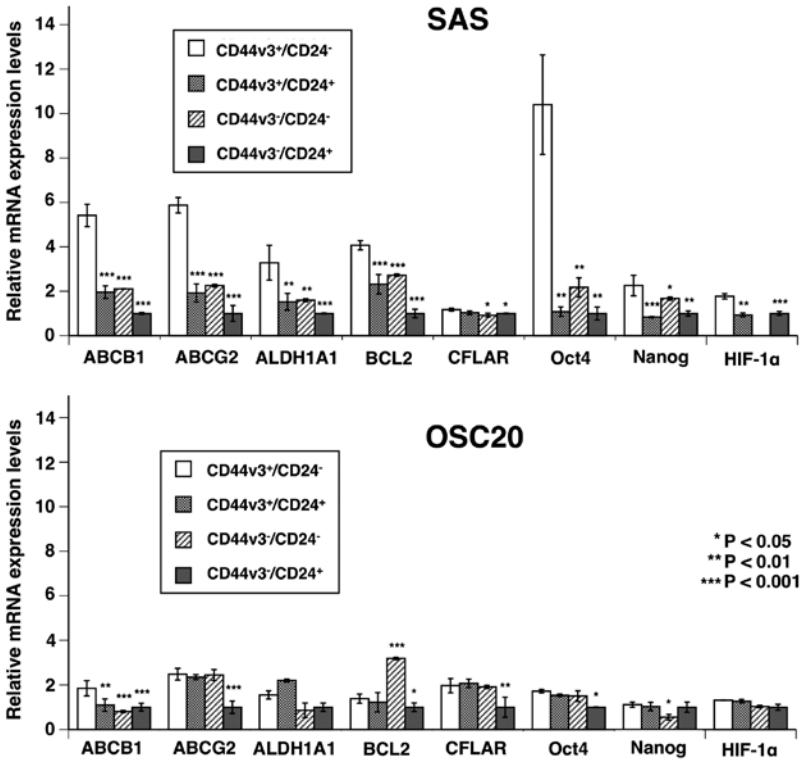

Figure 3. Quantification of mRNA expression of CSC-LC property-related genes in sorted cell fractions by real-time PCR. In SAS, CD44v3+/CD24- cells show significantly higher mRNA expression of transporter-related genes (ABCB1 and ABCG2), ALDH1A1, anti-apoptotic genes (BCL2) and self-replication genes (Oct-4 and Nanog) than the other fractions. The experiments were repeated at least three times for each cell line, and almost identical results were obtained (*P<0.05, **P<0.01, ***P<0.001).

Table II. Association of CD44v3 and CD24 immunoexpression with clinicopathological parameters in 50 OSCC patients.

		Invasive portion			Invasive portion		
		Immunoreactive score of CD44v3			Immunoreactive score of CD24		
Variables	Total no.	+	-	P-value	+	-	P-value
No. of patients	50	23	27		11	39	
Age (years)				0.969			0.705
≤65	14	6	8		2	12	
>65	36	17	19		9	27	
Gender				0.897			0.01
Male	32	15	17		3	29	
Female	18	8	10		8	10	
T classification				0.763			0.688
T1/T2	39	17	22		8	31	
T3/T4	11	6	5		3	8	
N classification				0.039			0.64
N0	43	17	26		9	34	
N1+N2	7	6	1		2	5	
Mode of invasion				0.575			0.938
1+2	13	6	7		3	10	
3	24	13	11		6	18	
4	13	4	9		2	11	
Differentiation				0.601			0.147
Well	34	17	17		5	29	
Moderate + poor	16	6	10		6	10	

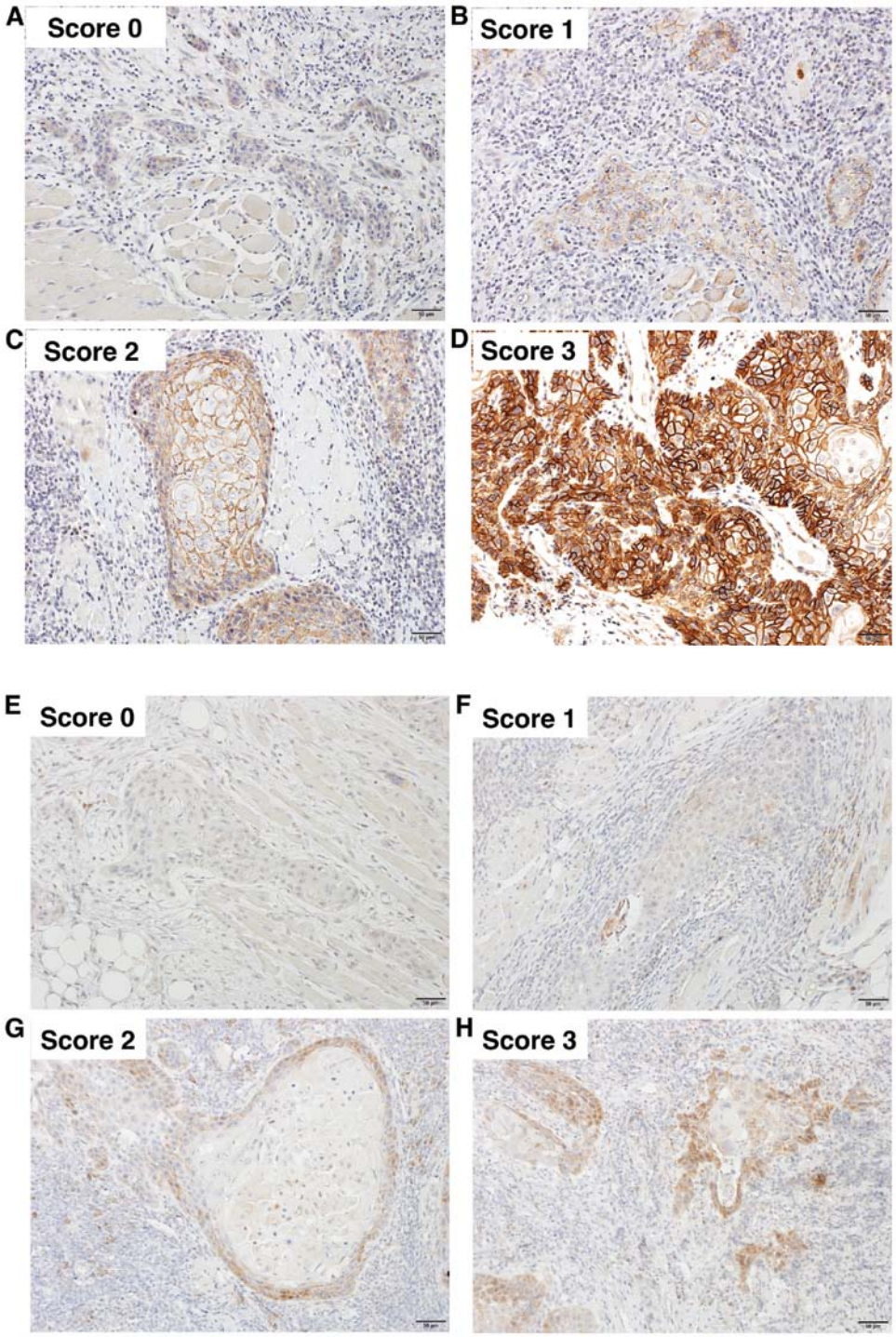

Figure 4. Immunostaining of (A-D) CD44v3 and (E-H) CD24 in OSCC tissues. The staining intensity was graded into 4 levels, i.e., 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive.

photomicrograph of each grade is shown in Fig. 4. CD44v3 and CD24 immunoscoring of the non-invasive portion and invasive portion is shown in Fig. 5. A significant difference was observed between the expression scores of CD24 in the non-invasive portion and those in the invasive portion ($P<0.01$) (Fig. 5).

There was no significant correlation between isolated CD44v3 and isolated CD24 immunoexpression or CD44v3/CD24 immunophenotypes and each clinicopathological parameter in the non-invasive portion (data not shown). The association between CD44v3 and CD24 expression in the invasive portion and clinicopathological parameters is summarized in Table II. CD44v3 expression was significantly correlated with lymph node metastasis ($P=0.039$), but not with age, gender, T stage, mode of invasion and tumor differentiation. CD24 expression was significantly correlated with gender ($P=0.01$), but not with the other clinicopathological parameter. The association between CD44v3/CD24 immunophenotypes and clinicopathological parameters in 50 OSCC patients is summarized in Table III. When the 50 cases were subdivided

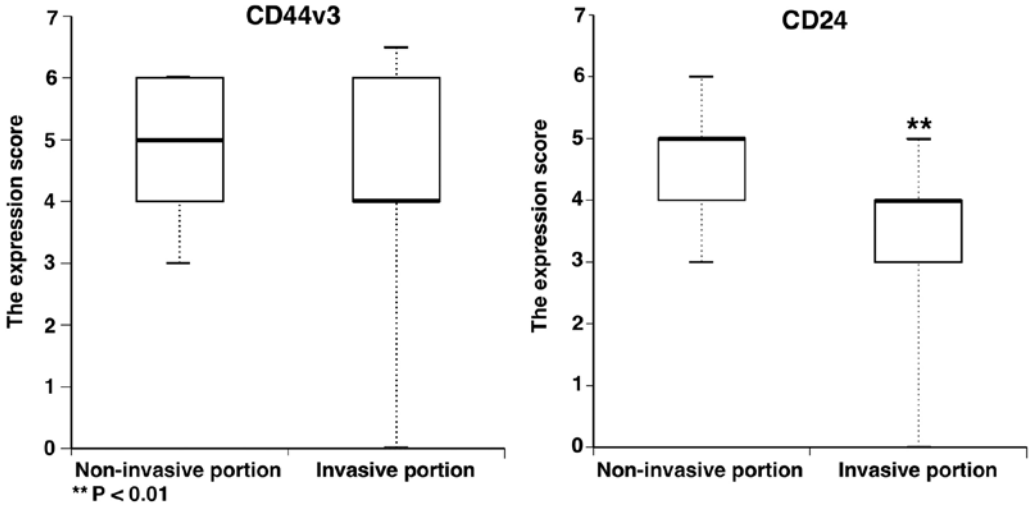

Figure 5. Immunostaining of CD44v3 and CD24 in the non-invasive portion and invasive portion. Box and Whisker plots of CD44v3 and CD24 immunoscoring of the non-invasive portion and invasive portion showing median (bold line) and 25th to 75th percentiles (**P<0.01).

Table III. Association of CD44v3/CD24 immunophenotype with clinicopathological parameters in 50 OSCC patients.

		Invasive portion				
		CD44v3/CD24 profile				
Variables	Total no	+/-	+/+	-/-	-/+	P-value
No. of patients	50	18	5	21	6	
Age (years)						0.82
≤65	14	6	0	6	2	
>65	36	12	5	15	4	
Gender						0.139
Male	32	13	2	16	1	
Female	18	5	3	5	5	
T classification						0.882
T1/T2	39	13	4	18	4	
T3/T4	11	5	1	3	2	
Nodal status						0.862
N0	43	14	4	19	6	
N1 + N2	7	4	1	2	0	
Mode of invasion						0.647
1 + 2	13	3	3	7	0	
3	24	11	2	7	4	
4	13	4	0	7	2	
Differentiation						0.505
Well	34	14	3	15	2	
Moderate + poor	16	4	2	6	4	

+/- cases, $CD44v3^+/CD24^-$; +/+ cases, $CD44v3^+/CD24^+$; -/- cases, $CD44v3^-/CD24^-$; -/+ cases, $CD44v3^-/CD24^+$.

into four groups i.e., $CD44v3^+/CD24^-$, $CD44v3^+/CD24^+$, $CD44v3^-/CD24^-$ and $CD44v3^-/CD24^+$ cases, the four immunophenotypes were not significantly correlated with each clinicopathological parameter.

Overall survival (OS) rates and CD44v3/CD24 immunophenotypes in the invasive portion. Kaplan Meier analysis established the relationship between CD44v3 expression in the invasive portion and OS (Fig. 6A). $CD44v3^+$ cases tended to show poor OS compared with $CD44v3^-$ cases (P=0.055). The relationship between CD24 expression in the invasive portion and OS is shown in Fig. 6B. There was no significant difference in OS between $CD24^+$ cases and $CD24^-$ cases. The Kaplan Meier curves for OS in OSCC patients that were subdivided into four groups according to CD44v3/CD24 immunophenotypes are shown in Fig. 6C. $CD44v3^+/CD24^-$ cases showed significantly worse OS than $CD44v3^+/CD24^-$ cases (P=0.029).

Discussion

In the present study, $CD44v3^+/CD24^-$ cell fractions in SAS and OSC20 were 10.7 and 24.1%, respectively. Although the $CD44v3^+/CD24^-$ cell fraction in SAS or OSC20 did not show a higher proliferative ability than each of the other fractions ($CD44v3^-/CD24^+$, $CD44v3^-/CD24^-$ and $CD44v3^-/CD24^+$ cells), the $CD44v3^+/CD24^-$ cell fraction showed significantly higher sphere forming ability, a higher CDDP or 5-FU resistance, suggesting that the $CD44v3^+/CD24^-$ cell fraction had CSC-LC properties in SAS or OSC20.

Previous studies have reported that CSC-LCs have drug transporter genes such as ABCB1 and ABCG2 (26,27). Our real-time PCR assay found that drug transporter genes such as ABCB1 and ABCG2 were significantly more highly expressed in the $CD44v3^+/CD24^-$ cell fraction than the other cell fractions in SAS. However, ABCB1 and ABCG2 are not major transporters of CDDP or 5-FU, and therefore, high expression of ABCB1 and ABCG2 is unlikely to be the explanation for the increased resistance to CDDP or 5-FU in SAS. Other studies have reported that CSC-LCs have anti-apoptotic and drug-resistant properties due to the expression of anti-apoptosis genes such as BCL2 and CFLAR (28), which contribute to the

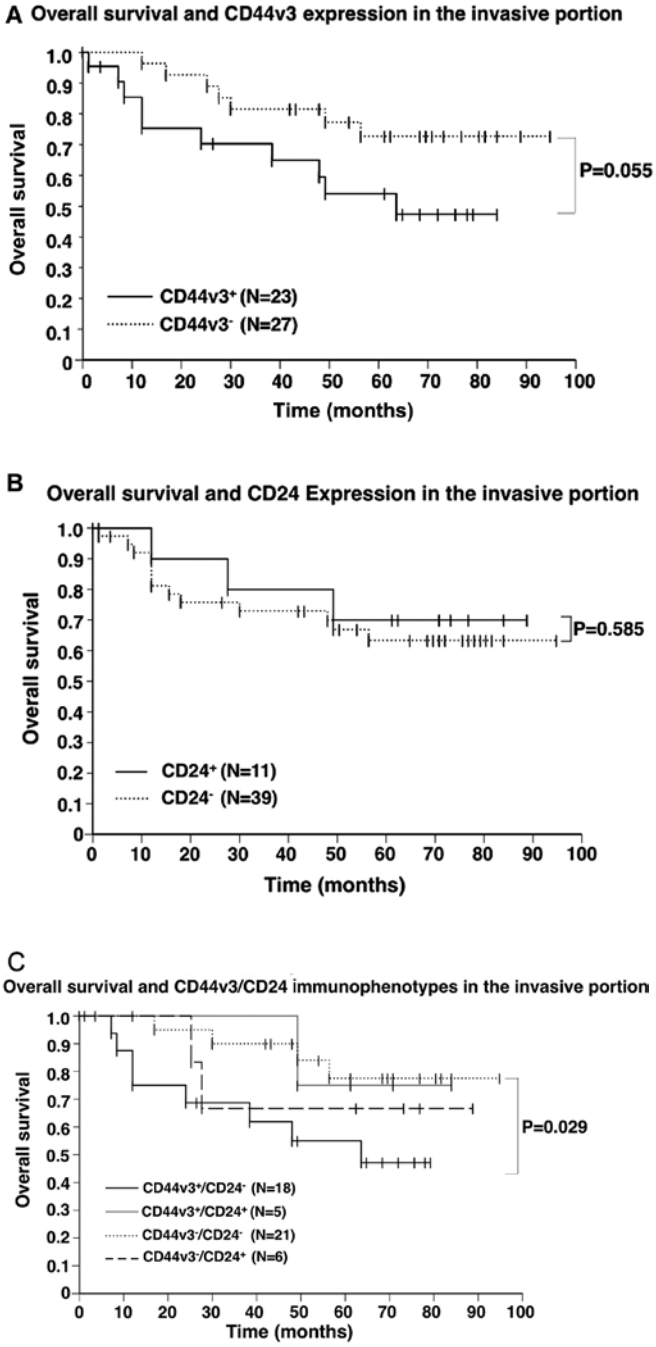

Figure 6. Overall survival (OS) rates and CD44v3/CD24 immunophenotypes in the invasive portion. (A) CD44v3$^+$ cases tended to show poor OS compared with CD44v3$^-$ cases (P=0.055). (B) There was no significant difference in OS between CD24$^+$ cases and CD24$^-$ cases (P=0.585). (C) The Kaplan Meier curves for OS in OSCC patients were subdivided into 4 groups according to CD44v3/CD24 immunophenotypes (CD44v3$^+$/CD24$^-$, CD44v3$^+$/CD24$^-$, CD44v3$^-$/CD24$^-$ and CD44v3$^-$/CD24$^+$). CD44v3$^+$/CD24$^-$ cases showed poor OS, and there was a significant difference between CD44v3$^+$/CD24$^-$ and CD44v3$^-$/CD24$^-$ cases (P=0.029).

emergence of CDDP or 5-FU resistance (29-32). Our real-time PCR assays found that anti-apoptotic genes such as BCL2 and CFLAR were more highly expressed in the CD44v3$^+$/CD24$^-$ cell fraction than the other cell fractions in SAS. These findings suggest that CD44v3$^+$/CD24$^-$ cells have anti-apoptotic effects; therefore, they probably showed higher CDDP or 5-FU resistance. Oct-4 and Nanog are associated with self-renewal capacity (14,33). Our real-time PCR assays also found that self-replication markers such as Oct4 or Nanog were significantly more highly expressed in the CD44v3$^+$/CD24$^-$ cell fraction than the other cell fractions in SAS.

Indeed, CD44v3$^+$/CD24$^-$ showed the highest sphere forming abilities compared with the other fractions in both cell lines. Sphere forming assay is a representative assay used to confirm self-renewal capacity *in vitro*. Collectively, these findings suggest that the CD44v3$^+$/CD24$^-$ cell fraction had a higher self-renewal capacity.

Before the presence of CSC was widely accepted in solid tumors, immunohistochemical studies on CD44, including its variant isoform such as CD44v3 and CD24 were conducted in various tumors, including OSCCs. However, the relationship between these markers and various clinicopathological findings was inconsistent in several previous studies (34-40). The present study demonstrated that CD44v3 expression in the invasive portion was slightly downregulated compared with the non-invasive portion. These findings are partially consistent with several studies on CD44 isoforms in OSCCs (36,41-43). Molecular cross-talk between tumor and host at the invasion front is probably associated with these alterations of adhesion molecules, such as CD44 isoforms (36,44,45). Our present study demonstrated CD24 expression in the invasive portion was significantly downregulated compared with expression in the non-invasive portion. In some reports, the upregulation of CD24 expression was associated with an early event of carcinogenesis in various carcinomas (35,39,46). In the present study, CD24 expression in the invasive portion was found in only 22% of cases and was not associated with prognosis. However, out of the cases without CD24 expression, the cases with unfavorable prognosis were enhanced when combined with CD44v3$^+$ expression. As we described before, immunohistochemical analyses on CD44 and/or CD24 were inconsistent. These inconsistent results may be due to extreme variations in tissue material, varying specificity of the numerous different antibodies used, and the diverse scoring and evaluation system applied (36).

CD44v3$^+$ cells showed a higher tumorigenicity *in vivo* assay compared with CD44v3$^-$ cells. These effects were almost independent of CD24 status. Also, immunohistochemical findings demonstrated that CD44v3 was more prognostic as a single marker in OSCCs. Collectively, these findings suggest that CD44v3 may be a more reliable marker for CSC-LC properties than CD24 as a single marker. The interaction of CD44 isoforms and CD24 is probably associated with each other directly or indirectly. Further studies on the association between CD44 isoforms and CD24 should be conducted to clarify the exact mechanisms.

In conclusion, the results suggest that the CD44v3$^+$/CD24$^-$ cell population shows CSC-LC properties in a human OSCC cell line. Additionally, we presented some evidence that isolated CD44v3 immunoexpression or CD44v3$^+$/CD24$^-$ immunophenotypes could give prognostic information associated with unfavorable clinical outcomes.

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References

1. Collins AT, Berry PA, Hyde C, Stower MJ and Maitland NJ: Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65: 10946-10951, 2005.
2. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, *et al*: Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 104: 10158-10163, 2007.
3. Kalish ED, Iida N, Moffat FL and Bourguignon LY: A new CD44V3-containing isoform is involved in tumor cell growth and migration during human breast carcinoma progression. Front Biosci 4: A1-A8, 1999.
4. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983-3988, 2003.
5. Ma F, Li H, Wang H, Shi X, Fan Y, Ding X, Lin C, Zhan Q, Qian H and Xu B: Enriched $CD44^+/CD24^-$ population drives the aggressive phenotypes presented in triple-negative breast cancer (TNBC). Cancer Lett 353: 153-159, 2014.
6. Hurt EM, Kawasaki BT, Klarmann GJ, Thomas SB and Farrar WL: $CD44^+CD24^-$ prostate cells are early cancer progenitor/stem cells that provide a model for patients with poor prognosis. Br J Cancer 98: 756-765, 2008.
7. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF and Simeone DM: Identification of pancreatic cancer stem cells. Cancer Res 67: 1030-1037, 2007.
8. Petersen PE: Oral cancer prevention and control - the approach of the World Health Organization. Oral Oncol 45: 454-460, 2009.
9. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R: Identification and expansion of human colon-cancer-initiating cells. Nature 445: 111-115, 2007.
10. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT and Jacks T: Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 121: 823-835, 2005.
11. Wang J, Guo LP, Chen LZ, Zeng YX and Lu SH: Identification of cancer stem cell-like side population cells in human nasopharyngeal carcinoma cell line. Cancer Res 67: 3716-3724, 2007.
12. Takaishi S, Okumura T, Tu S, Wang SS, Shibata W, Vigneshwaran R, Gordon SA, Shimada Y and Wang TC: Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27: 1006-1020, 2009.
13. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF and Ailles LE: Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 104: 973-978, 2007.
14. Chen C, Wei Y, Hummel M, Hoffmann TK, Gross M, Kaufmann AM and Albers AE: Evidence for epithelial-mesenchymal transition in cancer stem cells of head and neck squamous cell carcinoma. PLoS One 6: e16466, 2011.
15. Wang SJ, Wong G, de Heer AM, Xia W and Bourguignon LY: CD44 variant isoforms in head and neck squamous cell carcinoma progression. Laryngoscope 119: 1518-1530, 2009.
16. Bourguignon LY, Wong G, Earle C and Chen L: Hyaluronan-CD44v3 interaction with Oct4-Sox2-Nanog promotes miR-302 expression leading to self-renewal, clonal formation, and cisplatin resistance in cancer stem cells from head and neck squamous cell carcinoma. J Biol Chem 287: 32800-32824, 2012.
17. Pirruccello SJ and LeBien TW: The human B cell-associated antigen CD24 is a single chain sialoglycoprotein. J Immunol 136: 3779-3784, 1986.
18. Fischer GF, Majdic O, Gadd S and Knapp W: Signal transduction in lymphocytic and myeloid cells via CD24, a new member of phosphoinositol-anchored membrane molecules. J Immunol 144: 638-641, 1990.
19. Sano A, Kato H, Sakurai S, Sakai M, Tanaka N, Inose T, Saito K, Sohda M, Nakajima M, Nakajima T, *et al*: CD24 expression is a novel prognostic factor in esophageal squamous cell carcinoma. Ann Surg Oncol 16: 506-514, 2009.
20. Yang XR, Xu Y, Yu B, Zhou J, Li JC, Qiu SJ, Shi YH, Wang XY, Dai Z, Shi GM, *et al*: CD24 is a novel predictor for poor prognosis of hepatocellular carcinoma after surgery. Clin Cancer Res 15: 5518-5527, 2009.
21. Yeung TM, Gandhi SC, Wilding JL, Muschel R and Bodmer WF: Cancer stem cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci USA 107: 3722-3727, 2010.
22. Chen YC, Chen YW, Hsu HS, Tseng LM, Huang PI, Lu KH, Chen DT, Tai LK, Yung MC, Chang SC, *et al*: Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. Biochem Biophys Res Commun 385: 307-313, 2009.
23. Chiu CC, Lee LY, Li YC, Chen YJ, Lu YC, Li YL, Wang HM, Chang JT and Cheng AJ: Grp78 as a therapeutic target for refractory head-neck cancer with $CD24^-CD44^+$ stemness phenotype. Cancer Gene Ther 20: 606-615, 2013.
24. Hisaka T, Yano H, Ogasawara S, Momosaki S, Nishida N, Takemoto Y, Kojiro S, Katafuchi Y and Kojiro M: Interferon-alphaCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. J Hepatol 41: 782-789, 2004.
25. Yamamoto E, Kohama G, Sunakawa H, Iwai M and Hiratsuka H: Mode of invasion, bleomycin sensitivity, and clinical course in squamous cell carcinoma of the oral cavity. Cancer 51: 2175-2180, 1983.
26. Rizzo S, Hersey JM, Mellor P, Dai W, Santos-Silva A, Liber D, Luk L, Titley I, Carden CP, Box G, *et al*: Ovarian cancer stem cell-like side populations are enriched following chemotherapy and overexpress EZH2. Mol Cancer Ther 10: 325-335, 2011.
27. Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, Goodell MA and Brenner MK: A distinct 'side population' of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 101: 14228-14233, 2004.
28. Yajima T, Ochiai H, Uchiyama T, Takano N, Shibahara T and Azuma T: Resistance to cytotoxic chemotherapy-induced apoptosis in side population cells of human oral squamous cell carcinoma cell line Ho-1-N-1. Int J Oncol 35: 273-280, 2009.
29. Yang Z, Liu Y, Liao J, Gong C, Sun C, Zhou X, Wei X, Zhang T, Gao Q, Ma D, *et al*: Quercetin induces endoplasmic reticulum stress to enhance cDDP cytotoxicity in ovarian cancer: Involvement of STAT3 signaling. FEBS J 282: 1111-1125, 2015.
30. Karaayvaz M, Zhai H and Ju J: miR-129 promotes apoptosis and enhances chemosensitivity to 5-fluorouracil in colorectal cancer. Cell Death Dis 4: e659, 2013.
31. Longley DB, Wilson TR, McEwan M, Allen WL, McDermott U, Galligan L and Johnston PG: c-FLIP inhibits chemotherapy-induced colorectal cancer cell death. Oncogene 25: 838-848, 2006.
32. Micheau O, Solary E, Hammann A and Dimanche-Boitrel MT: Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. J Biol Chem 274: 7987-7992, 1999.
33. Murakami S, Ninomiya W, Sakamoto E, Shibata T, Akiyama H and Tashiro F: SRY and OCT4 are required for the acquisition of cancer stem cell-like properties and are potential differentiation therapy targets. Stem Cells 33: 2652-2663, 2015.
34. Oliveira LR, Oliveira-Costa JP, Araujo IM, Soave DF, Zanetti JS, Soares FA, Zucoloto S and Ribeiro-Silva A: Cancer stem cell immunophenotypes in oral squamous cell carcinoma. J Oral Pathol Med 40: 135-142, 2011.
35. Abdulmajeed AA, Dalley AJ and Farah CS: Putative cancer stem cell marker expression in oral epithelial dysplasia and squamous cell carcinoma. J Oral Pathol Med 42: 755-760, 2013.
36. Bánkfalvi A, Krassort M, Buchwalow IB, Végh A, Felszeghy E and Piffkó J: Gains and losses of adhesion molecules (CD44, E-cadherin, and beta-catenin) during oral carcinogenesis and tumour progression. J Pathol 198: 343-351, 2002.
37. Choi D, Lee HW, Hur KY, Kim JJ, Park GS, Jang SH, Song YS, Jang KS and Paik SS: Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma. World J Gastroenterol 15: 2258-2264, 2009.
38. Kristiansen G, Denkert C, Schlüns K, Dahl E, Pilarsky C and Hauptmann S: CD24 is expressed in ovarian cancer and is a new independent prognostic marker of patient survival. Am J Pathol 161: 1215-1221, 2002.
39. Bircan S, Kapucuoglu N, Baspinar S, Inan G and Candir O: CD24 expression in ductal carcinoma in situ and invasive ductal carcinoma of breast: An immunohistochemistry-based pilot study. Pathol Res Pract 202: 569-576, 2006.
40. Darwish NS, Kim MA, Chang MS, Lee HS, Lee BL, Kim YI and Kim WH: Prognostic significance of CD24 expression in gastric carcinoma. Cancer Res Treat 36: 298-302, 2004.
41. Herold-Mende C, Seiter S, Born AI, Patzelt E, Schupp M, Zöller J, Bosch FX and Zöller M: Expression of CD44 splice variants in squamous epithelia and squamous cell carcinomas of the head and neck. J Pathol 179: 66-73, 1996.

42. Fonseca I, Pereira T, Rosa-Santos J and Soares J: Expression of CD44 isoforms in squamous cell carcinoma of the border of the tongue: A correlation with histological grade, pattern of stromal invasion, and cell differentiation. J Surg Oncol 76: 115-120, 2001.
43. Masuda M, Kuratomi Y, Shiratsuchi H, Nakashima T, Naonobu K and Komiyama S: Decreased CD44H expression in early-stage tongue carcinoma associates with late nodal metastases following interstitial brachytherapy. Head Neck 22: 662-665, 2000.
44. Williams HK, Sanders DS, Jankowski JA, Landini G and Brown AM: Expression of cadherins and catenins in oral epithelial dysplasia and squamous cell carcinoma. J Oral Pathol Med 27: 308-317, 1998.
45. Bànkfalvi A and Piffkò J: Prognostic and predictive factors in oral cancer: The role of the invasive tumour front. J Oral Pathol Med 29: 291-298, 2000.
46. Sagiv E, Memeo L, Karin A, Kazanov D, Jacob-Hirsch J, Mansukhani M, Rechavi G, Hibshoosh H and Arber N: CD24 is a new oncogene, early at the multistep process of colorectal cancer carcinogenesis. Gastroenterology 131: 630-639, 2006.