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+/CD24- immunophenotypes could give prognostic information associated with unfavorable clinical outcomes.

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).

CD44 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 CD44 is associated with invasion, metastasis and tumor differentiation (19,20), whether CD44 expression is upregulated or downregulated with tumor invasion remains unclear. CD44 has also been studied in combination with CD24. 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).

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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., Gaithersburg, MD, USA). OSC20 was grown in Eagle's minimal essential medium (EMEM; Gibco, BRL/Life Technologies Inc.) with 5% FBS. Cells were cultured in an atmosphere of 5% CO2 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 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 CO2 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-2-yl)-2,5-dimethyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon, Temecula, USA) using ultra-low attachment 6-well plates (Corning Inc., Corning, NY, USA) for 1 week. Sphere formation was assessed by using two human OSCC cell lines and 50 human OSCC tissues.

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 (1x10³, 1x10⁴, or 1x10⁵) 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, and 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...
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.

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- 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).

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- cells, 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
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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).

Table I. Tumorigenicity of sorted cell fractions in SAS.

| Injected cell number | CD44v3+/CD24- | CD44v3+/CD24+ | CD44v3-/CD24- | CD44v3-/CD24+ |
|----------------------|---------------|---------------|---------------|---------------|
| 1x10³                | 0/5 (0%)      | 0/5 (0%)      | 0/5 (0%)      | 0/5 (0%)      |
| 1x10⁴                | 2/5 (40%)     | 2/5 (40%)     | 0/5 (0%)      | 0/5 (0%)      |
| 1x10⁵                | 2/5 (40%)     | 3/5 (60%)     | 1/5 (20%)     | 2/5 (40%)     |

Tumorigenicity assays in vivo in sorted cell fractions in SAS. Injection of 1x10³ cells from all of the cell fractions produced no tumors in NOD/SCID mice. In contrast, two mice that received 1x10⁴ CD44v3+/CD24- cells or CD44v3+/CD24+ cells developed tumors 8 weeks after the inoculation. In addition, injection of 1x10⁵ 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<0.01, "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<0.01).
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.

| Variables               | Total no. | Invasive portion | Immunoreactive score of CD44v3 | P-value | Invasive portion | Immunoreactive score of CD24 | P-value |
|-------------------------|-----------|------------------|--------------------------------|---------|------------------|--------------------------------|---------|
| No. of patients         | 50        |                  |                                 |         |                  |                                |         |
| Age (years)             |           |                  |                                 |         |                  |                                |         |
| ≤65                     | 14        |                  |                                 | 0.969   |                  |                                | 0.705   |
| >65                     | 36        |                  |                                 |         |                  |                                |         |
| Gender                  |           |                  |                                 | 0.897   |                  |                                | 0.01    |
| Male                    | 32        |                  |                                 |         |                  |                                |         |
| Female                  | 18        |                  |                                 |         |                  |                                |         |
| T classification        |           |                  |                                 | 0.763   |                  |                                | 0.688   |
| T1/T2                   | 39        |                  |                                 |         |                  |                                |         |
| T3/T4                   | 11        |                  |                                 |         |                  |                                |         |
| N classification        |           |                  |                                 | 0.039   |                  |                                | 0.64    |
| N0                      | 43        |                  |                                 |         |                  |                                |         |
| N1+N2                   | 7         |                  |                                 |         |                  |                                |         |
| Mode of invasion        |           |                  |                                 | 0.575   |                  |                                | 0.938   |
| 1+2                     | 13        |                  |                                 |         |                  |                                |         |
| 3                       | 24        |                  |                                 |         |                  |                                |         |
| 4                       | 13        |                  |                                 |         |                  |                                |         |
| Differentiation         |           |                  |                                 | 0.601   |                  |                                | 0.147   |
| Well                    | 34        |                  |                                 |         |                  |                                |         |
| Moderate + poor         | 16        |                  |                                 |         |                  |                                |         |
The association between CD44v3 and CD24 immunoreactivity 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...
The expression score

Non-invasive portion | Invasive portion
---|---
CD44v3 | | 0.05

The expression score

Non-invasive portion | Invasive portion
---|---
CD24 | | **P<0.01**

Figure 5. Immunostaining of CD44v3 and CD24 in the non-invasive portion and invasive portion. Box and Whisker plots of CD44v3 and CD24 immunostaining of the non-invasive portion and invasive portion showing median (bold line) and 25th to 75th percentiles (**P<0.01).
The emergence of CDDP or 5-FU resistance (29-32). Our real-time PCR assays also 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.

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 immunophenotype or CD44v3+/CD24- immunophenotypes could give prognostic information associated with unfavorable clinical outcomes.

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