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Gene Expression of Cancer Stem Cell in Oral Squamous Cell Carcinoma

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Abstract: Oral squamous cell carcinoma (OSCC), like many solid tumors, contains a heterogeneous population of cancer cells. Recent data suggest that a rare subpopulation of cancer cells, known as cancer stem cells (CSCs), is capable of initiating, maintaining, and expanding the growth of tumors. Identification and characterization of CSCs from OSCC would facilitate the monitoring, therapy, or prevention of this cancer. CD133 is considered a marker molecule for CSCs; however, its role in OSCC is yet to be determined. In this study, we isolated CD133-positive cells from OSCC cell lines using a magnetic-activated cell sorter (MACS). The differential expression of genes between OSCC/CD133-positive cells and parental cells was determined by polymerase chain reaction (PCR). Up-regulated genes (CD133-positive vs. parental cells) included ALDH 1, Keratin15 (Krt15), SOX2, and WNT 1, while the down-regulated genes included Fgfr1 and Pparg. Additionally, immunohistochemical analysis revealed that expression of Krt15 and SOX2 was localized to cancer cells of OSCC specimens. Their elevated expression levels were detected in poorly differentiated and chemo-resistant OSCC. Our results possibly demonstrate that CD133-positive cells, when compared with parental cells, are a more concentrated population of CSCs in OSCC.

Key words: oral squamous cell carcinoma, cancer stem cell, CD133, keratin 15, side population.

Stem cells are defined as pluripotent cells with the ability of self-renewal. Stem cells are a major population that have the ability to maintain the homeostasis and function of organs, and can be classified into two groups. The first of these is the embryonic stem (ES) cell. The ES cell exists for initial embryo generation, and can differentiate into all cell types. The other is a somatic stem cell, existing in each organ after organogenesis, which can self-renew and maintain tissue homeostasis. While there are tissue-specific stem cells in each organ, their respective activities are known to be different.[1~4)]

Somatic stem cells have been reported to exist in the intestinal epithelium, epidermis, mesenchyme, the central nervous system and many other organs.[5,6)] Mesenchymal stem cells are well-known stem cells with the ability to differentiate into adipocytes, osteoblasts, cartilage cells, and bone marrow interstitial cells; they are expected to be a promising source of stem cells in regenerative medicine.[7)] Surprisingly, even central nerve tissue, which had been believed to possess no stem cells, has now been shown to possess stem cell activity. When the activity of somatic stem cells is compared with ES cells, it can be observed that differentiation capacity is much lower.

It has been reported that each stem cell maintains its self-renewal and differentiation capacity through niche formation with neighboring cells.[8)] These niches form a microenvironment to maintain the undifferentiated status of the stem cell; they consist of adhesion molecules and cytokines including N-cadherin, VE-cadherin and TGF-β. Thus, the function of stem cells, with their various roles in the cell cycle, cell division, and cell kinetics of apoptosis, are regulated by a microenvironment and can remain in a quiescent state for a prolonged period.[9)]

Tumors also contain stem cells, designated cancer stem cells (CSCs). The tumorigenic characteristics of CSCs are similar to those of normal stem cells, with an early report

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from Dick et al. indicating that in human acute myeloid leukemia, the $CD34^{+}CD38^{-}$ cells were leukemia-initiating cells.[10] Subsequently, the existence of CSCs were also noticed in solid cancers, such as breast cancer, brain tumors, prostate cancer, esophageal cancer and liver cancer. Indeed, there is a hierarchy between cancer-initiating/CSCs and other cancer cells, but it remains uncertain whether CSCs are involved in carcinogenesis, invasion and metastasis. Two theories have been proposed concerning the relationship between CSCs and carcinogenesis. The first is that normal stem cells acquire the properties of tumor cells through some unknown mechanism(s); the second is that a subset of tumor/cancer cells acquire(s) the properties of stem cells (Fig. 1). There is a possibility that tumor growth and metastasis are controlled by CSCs, which constitute a small population of heterogeneous cancer cells. If so, cancer therapy should be performed to identify and eliminate CSCs effectively (Fig. 2).

In this study, we used two methods to identify CSCs in OSCC. Side population (SP) assays and purification of CD133-positive cells by flow cytometry were conducted as both methods have been used previously to isolate CSCs.[11] Furthermore, the gene expression profile of CD133-positive cancer cells was examined.

Materials and Methods

Cell lines

Oral squamous cell carcinoma (OSCC) cell lines, Tosca23S, 24, and 55 were established by Tachikawa et al.[12] DMEM/Ham's F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cell lines were incubated at 37℃ in a humidified atmosphere with 5% CO_2. Cells were routinely maintained in 10 cm cell culture dish.

SP analysis

The suspension cells were labeled with Hoechst 33342 dye using the methods described by Goodell et al. with modifications.[13] Briefly, cells dissociated with 0.25% trypsin were resuspended in pre-warmed DMEM with 2% FSC and 10 mM HEPES buffer. Hoechst 33342 dye was added at final concentration of 2.5 μg /mL in the presence or absence of an ABC transporter inhibitor, reserpin, reserpin and the cells were incubated 37℃ for 30 min with shaking.[6,7] At the end of the incubation, the cells were washed with cold phosphate-buffered saline (PBS) containing 2% FBS, centrifuged at 4℃, and resuspended in cold PBS containing 2% FBS. Propidium iodide (PI) at a final concentration of 1 μg/mL was added to the cells to gate viable cells, followed by filtration through a 35-μm cell strainer to obtain single cell suspension before sorting.

Analysis and sorting were performed using FACS Vantage (Becton Dickinson). The Hoechst33342 dye was excited at 350 nm and its fluorescence was measured with both 424/44 nm (Hoechst blue) and 585/42 nm (Hoechst red) filters.

Magnetic-activated cell sorting (MACS)

Tosca23S cells (parental cell) were dissociated and resuspended in PBS containing 0.5% bovine serum albumin and 2 mM EDTA. Cells were labeled with anti-CD133 antibody (mouse IgG1, Military Biotec) and MACS MS column (Military Biotec).[8~10] All the procedures were carried out according to the manufacturer's instruction. CD133-positive Tosca 23S fractions were resuspended in DMEM containing 10% FBS and 1% penicillin/streptomycin.

Immunohistochemistry on cultured cells

CD133-positive Tosca23S cells and parental cells were cultured in four-chamber polystyrene tissue culture slides (Becton Dickinson, Montain) until sub confluent and washed in TBS, fixed in 10% PFA/1% Triton X-100 for 5 min. After rinsing in 0.01 M Tris buffered saline, pH7.4 (TBS), cultured cells were incubated with rabbit polyclonal anti-CD133 antibody (Santa Cruz, MA, USA), followed by incubation the cells were incubated with peroxidase-labeled polymer from the EnVision+system (DAKO), subsequently color reactions were obtained with 3,3-diaminobenzidine (DAKO) and the cells were counterstained with hematoxylin for the identification of nuclei.

Real-time quantitative RT-PCR

To assess the expression of stem cell related genes,

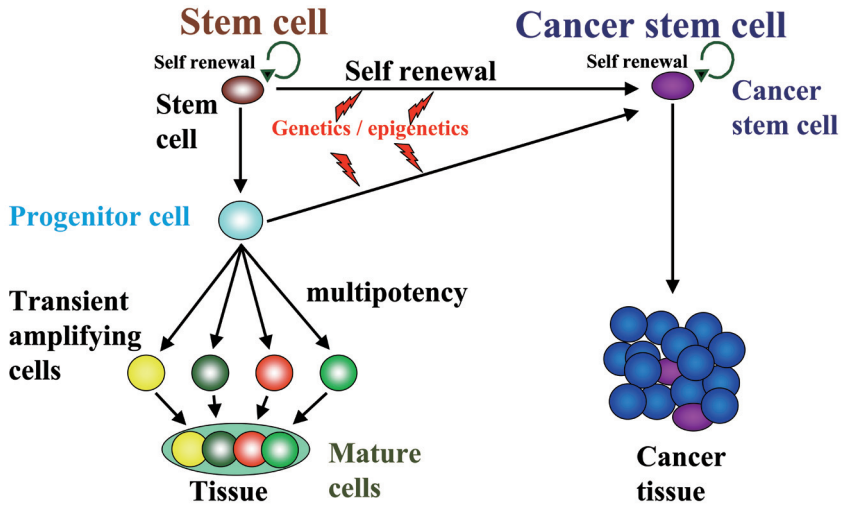

Fig. 1 Normal tissues arise from a central stem cell that grows and differentiates to create progenitor and mature cell populations. Normal stem cells possess multilineage potential and extensive proliferative capacity. Cancer stem cells arise by means of a mutation in normal stem cells or progenitor cells, and subsequently grow and differentiate to create primary tumors. Like normal stem cells, cancer stem cells can self-renew, give rise to heterogeneous populations of daughter cells, and proliferate extensively.

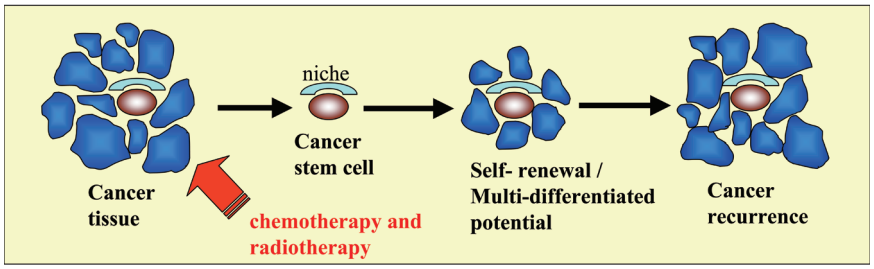

Fig. 2 Cancer stem cell/carcinogenesis concepts. The concept of cancer stem cells varies in different contexts. For example, cancer stem cells can be the source of all the malignant cells in a primary tumor and compose the small reservoir of drug-resistant cells that are responsible for relapse after chemotherapy induced remission.

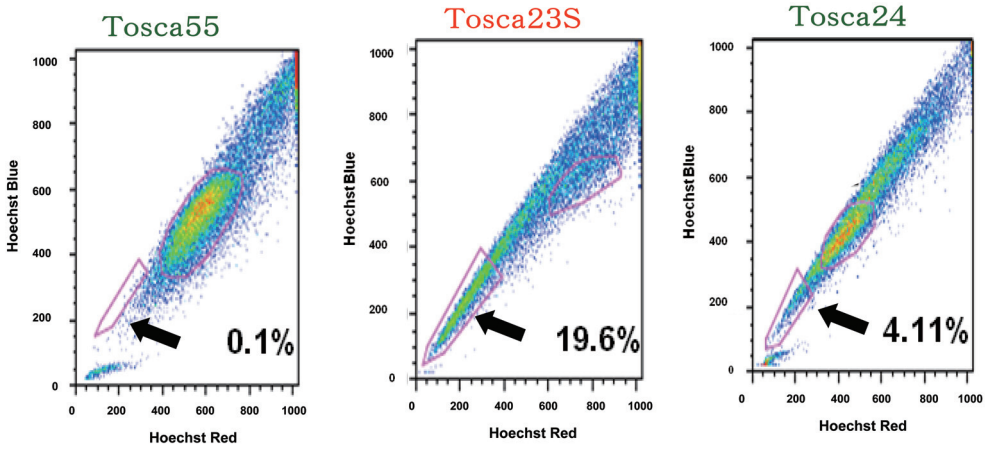

Fig. 3 SP analysis. Flow cytometry analysis with Hoecst33342 staining demonstrated that Tosca24, Tosca23S and Tosca55 cells included SP cells in 4%, 20% and 0.055%, respectively.

real-time quantitative RT-PCR was performed. We used the commercially available stem cell RT^2 Profiler^{TM} PCR Array (Super Array Bioscience Corp).

Total RNA was isolated from Tosca23S and CD133-positive Tosca23S cells using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The diluted first strand cDNA synthesis reaction was mixed with SuperArray PCR master mix, and loaded onto the 96-Well(Each array consisted genes of a panels of 96 primer sets of 84 stem cell pathway genes, plus five houskeepig genes and three RNA and PCR quality control) RT^2 Profiler^{TM} PCR Array (SuperArray, Inc., Frederick, MD, USA). Reactions were carried out using an ABI Prism 7000 Seuence Detection System (Applied Biosystems). Real-time PCR was performed by heating the plate at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min. The data were analyzed with ΔΔCt method according to the manufacturer's manual.

Immunohistochemistry staining of Krt 15, Nestin, and SOX2 in oral cancer patients

The human OSCC specimens were obtained with consent from patients who underwent surgery at the Department of Oral and Maxillofacial Surgery, Dental Hospital, Showa University, following local ethics committee guideline, to confirm the PCR results, immunohistochemical analysis was carried out with frozen section of normal epithelium, epithelial dysplasia and squamous cell carcinoma.

Frozen sections (4 μm thickness) were fixed with 4% PFA for 5 min. After rinsing with TBS, the sections were incubated with anti-cytokeratin 15 antibody (Novus Biological, Inc.), and anti-Sox antibody (ZYMED Laboratories), subsequently incubated with peroidaselabeled polymer from the EnVision+system(DAKO). Color reactions were obtained with 3,3-diaminobenzidine (DAKO), followed by counterstain with hematoxylin for the identification of nuclei.

Table 1 Up-regulated genes (selected).

Symbol(Name)	Fold Up-Regulation
ALDH1A1 (Aldehyde dehydrogenase 1 family, member A1)	8.71
KRT15 (Keratin 15)	5.53
SOX2 (SRY (sex determing region Y)-box 2)	5.40
FRAT1 (Frequentiy rearranged in advanced T-cell lymphomas)	4.81
ACAN (Aggrecan)	3.86
BMP3 (Bone morphogenetic protein 3)	3.31
WNT1 (Wingless-type MMTV integration site family, member 1)	3.07
GDF3 (Growth differentiation facter 3)	2.88
FGF3 (Fibroblast growth factor (murine mammary tumor virus integration site (vA-int-2) oncogene hamolog))	2.84
IGF1 (Insulin-like growth factor 1 (somatomedin C))	2.80
CD8B (CD8b molecule)	2.54
APC (Adenomatosis polyposis coli)	2.48
ALDH2 (aldehyde dehydrogenese 2 family (mitochondoria))	2.35
ACTC1 (Actin, alpha, cardiac muscle 1)	2.34
ALP (IAlkalie phosphatase, intestinal)	2.34
ASCL2 (Achaete-scute complex homolog 2)	2.34
CD4 (CD4 molecule)	2.34
CD8A (CD8a molecule)	2.34
CXCL12 (Chemokjne(C-X-Cmtif) lingand 12 (stromal cell-derived factor 1))	2.34
DHH (Desert hedgehog homolog)	2.34
DLL3 (Delta-like 3)	2.34
DTX1 (Deltex homolog 1)	2.34
FGF4 (Fibrablast growth facter 4 (heparin secretory transtanhing protein 1, Kaposi sarcorna))	2.34
GDF2 (Growth differentiation facter 2)	2.34
GJB1 (Gap jnction protein, alpha 1, 43 kDa)	2.34
PDX1 (Pancreatic and doudenum homeobox 1)	2.34

Table 2 Down-regulated genes (selected).

Symbol (Name)	Fold Down-Regulation
FGFR1 (Fibreblast growth factor receptor 1 (fms-reelated tyrosine kinase 2, Pfelffer syndrame)	0.98
MME (Membrane metallo-endopeptidase)	0.95
PPARG (Peroxisome proliferator-activated receptor delta)	0.92
CCND1 (Cyclin D1)	0.88
MSX1 (Msh homolog 1)	0.86
HPRT1 (Hypoxanthin phosphohbosyltransferase 1(Lesch-Nyllan syndrome))	0.83
COL2A1 (Collagen, type II, alpha I (primary osleoarthritis, spondyioeplphyseal dysplasia, congeital))	0.80
ACTB (Actin, beta)	0.75
CCNE1 (Cyclin E1)	0.74
ABCG2 (ATP-binding cassde, sub-family G (WHITE), member 2)	0.74
CDH2 (Cadherin 2, type1, N-cadherin(neuronal))	0.73
MYC (V-myc myelocytomatosis viral oncogene homoiog)	0.70
OPRS1 (Opoid receptor, sigma 1)	0.69
CCND2 (Cyclin D2)	0.68
CDC2 (Cell division cycle 2. Gl to S and G2 to M)	0.65
CD44 (CD44 molecule(indian blood group))	0.60
CCNA2 (Cyclin A2)	0.53
GJA1 (Gap juncton protein. alpha I . 43kDa)	0.44
FOXA2 (Forkh ead box A2) (Forhead box A2)	0.41
GJB2 (Gap juncton protein. beta I. 32 kDa)	0.39
GAPDH (Glyceralhyde-3-phosphate dehydrogenase)	0.33
TUBB3 (Tubulin, beta 3)	0.23
COL1A1 (Collagen, type 1, alpha 1)	0.20

Results

SP cells in OSCC cell lines

Flow cytometry analysis with Hoechst 33342 staining demonstrated that 4%, 20%, and 0.05% of the cells in Tosca24, Tosca23S and Tosca55 cell lines, respectively, were SP cells (Fig. 3).

Separation of CD133-positive Tosca23S cells

The Tosca23S cell line, which showed the highest proportion of SP cells, was analyzed because the composition of the SP is expected to reflect the frequency of CSCs. The CD133-positive Tosca23S cells were separated using a cell sorter (BECK MAN COULTER, Z1 coulter). The concentration of Tosca23S cells before separation was 3.6×10^6 cells/mL, with approximately 20% of these cells CD133-positive.

Gene expression analysis of CD133-positive Tosca23S cells

From our gene expression analysis, it was apparent that 38 genes were up-regulated and four were down-regulated (Table 1, 2). The up-regulated genes were associated with various roles including self-renewal, cell-cell communication, cell maintenance, signaling pathways and many other functions (Table 1). Of the up-regulated genes, there were self-renewal marker (NEUROG2, SOX1, SOX2) which is stem cell specific maker, cytokines and growth factor (BMP3,FGF3, FGF4, GDF2, GDF3, IGF1), genes regulating cell-cell communication (DHH, GJB1), cell adhesion molecules (APC, CDH1, COL9A1, CXCL12), metabolic markers (ALDH1A1, ALDH2), embryonic cell lineage markers (ASCL2, PDX1 (IPF1), KRT15, MYOD1), hematopoietic cell lineage markers (CD3D, CD4, CD8D), mesenchymal, cell lineage markers (ACAN(AGC1), ALPI) and neural cell lineage markers (NCAM1) which is stem cell differentiation maker, Notch Pathway (DLL3, DTX1, NOTCH2) and Wnt Pathway (ADAR, BTRC, FRAT1, WNT1) which is signaling pathways important for Stem cell maintenance (Table 1). Of the down-regulated genes (Table 2), these were related to the regulation of cell-cell communication (GJB2), an embryonic cell lineage marker (FOXA2), a mesenchymal cell lineage marker (COL1A1), and a stem cell-specific neural cell lineage marker (TUBB3).

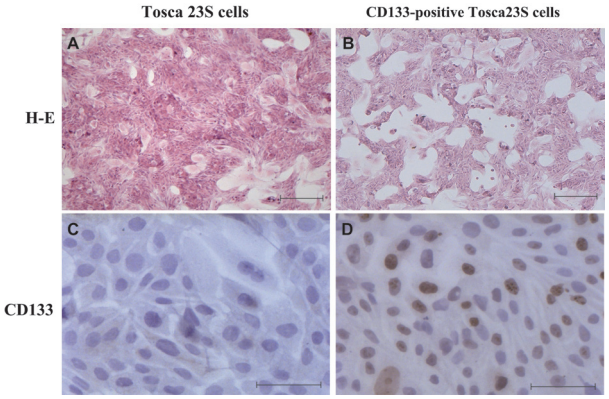

Fig. 4 Tosca 23S cells (A, C) and CD133-posittive Tosca23S cells (B, D) were cultured with DMEM containing 10%FBS and 1% penicillin/streptomycin. These cell lines were cultured in a humidified atmosphere with 5% CO_2. Cells were routinely maintained in four-chamber polystyrene tissue culture slides. After being in culture for 3 weeks, CD133-positive Tosca23S cells formed sphere-like bodies (B). (C, D): CD133-positive Tosca23S cells and Tosca23S cells were cultured in four-chamber polystyrene tissue culture slides until sub confluent and washed with TBS, fixed in 10% PFA/1% Triton X-100 for 5 min. After rinsing in TBS, cultured cells were incubated with a rabbit polyclonal anti-CD133 antibody followed by incubation with peroxidase-labeled polymer from the EnVision+system (DAKO), subsequently color reactions were obtained with 3,3-diaminobenzidine (DAB) and the cells were counterstained with hematoxylin for the identification of nuclei. The higher expression of CD133 was confirmed in CD133-positive Tosca23S cells (C, D) (scale bar; A. B: 500 μm, C. D: 50 μm).

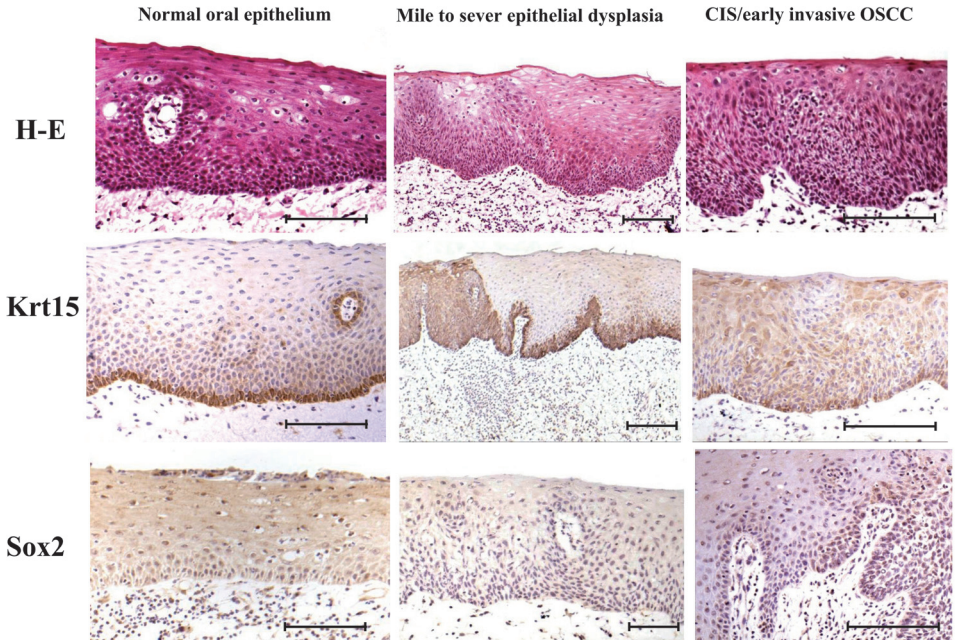

Fig. 5 Strong positive stainings for Krt15 were found in only basal cells of normal epithelium (left panel) and prickle cells in epithelial dysplasia (center panel). On the contrary, in CIS and SCC, the expression of Krt15 diminished except for the peripheral part of carcinoma foci in SCC (right panel) (scale bar; 250 μm).

Immunohistochemistry on cultured cells and OSCC specimens

Tosca23S cells proliferated rapidly. During the early stages of culture, CD133-positive Tosca23S cells formed small, round colonies, and then formed sheet-like structures as the culture progressed (Fig. 4 A, B). The level of CD133 expression was increased in CD133-positive Tosca23S cells as compared with parental cells

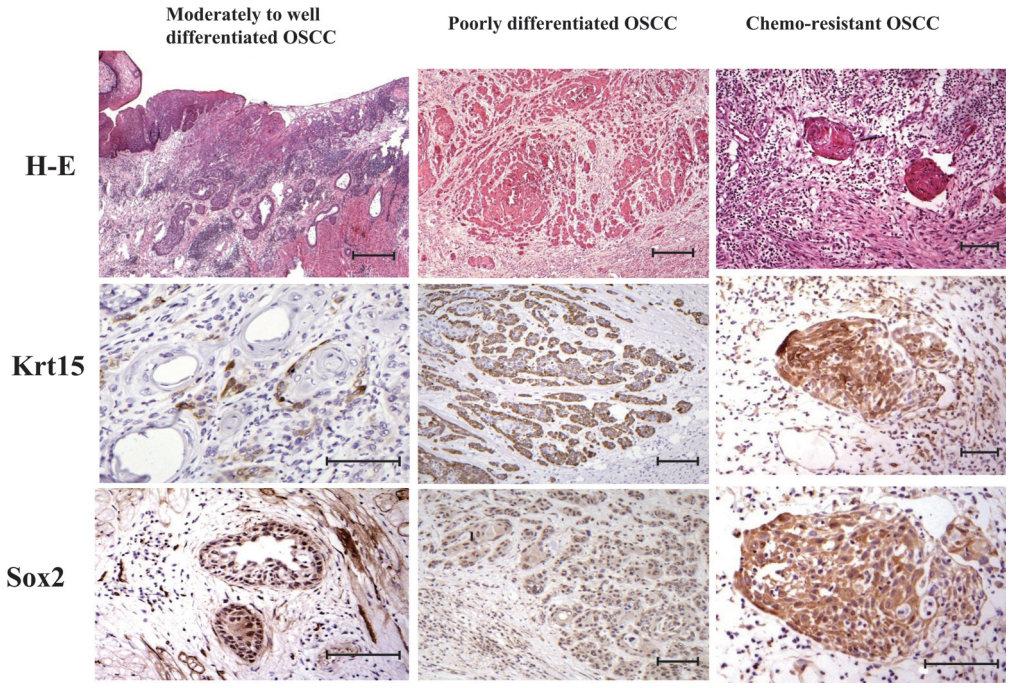

Fig. 6 In CIS and SCC, the expression of Krt15 diminished except for the peripheral part of carcinoma foci in SCC (left panel). On the other hand, poorly differentiated squamous cell carcinoma were positively stained (center panel). Moreover, positive stainings for Krt15 and SOX2 increased in most of cancer cells in OSCC specimens, which showed low sensitivity to chemotherapy (right panel). (scale bar; left panel: top 1 μm, middle and bottom: 50 μm, center panel: 50 μm, right panel: 1 μm).

(Fig. 4. C, D).

Strong positive staining for Krt15 was observed in basal cells of the normal epithelium and spread to prickle cells in the epithelial dysplasia (Fig. 5). However, for a carcinoma *in situ* (CIS) and squamous cell carcinomas (SCCs), the expression of Krt15 was localized to the peripheral part of the cancer. In poorly differentiated SCCs, there was some positive staining for these markers. Moreover, positive staining for Krt15 and SOX2 in cells was localized to the majority of the cancer cells in OSCC specimens obtained from patients with resistance to chemotherapy (Fig. 6).

Discussion

In this study, the SP of OSCCs was separated using flow cytometry in conjunction with Hoechst3342 staining. The SP cells in Tosca23S, Tosca55, and Tosca24 composed about 20%, 0.05%, and 4% of the total population, respectively. The SP cells from the bone marrow were an almost uniform cell population, being $CD34^{-}$, c-Kit^{+} and Lin^{-} with high hematopoietic activity.[14] The proportion of SP cells isolated from each OSCC line differed. The CD133-positive Tosca23S cells comprised approximately 20% of SP cells. These CD133-positive Tosca23S cells formed a sheet-like structure and exhibited rapid growth.

Our gene expression analysis revealed that 38 genes were up-regulated and 4 were down-regulated (Table 1～3). Aldehyde-dehydrogenase (ALDH) was one of the up regulated genes. It has been previously reported that ALDH is expressed in various stem cells, such as hematopoietic, corneal, and epidermal stem cells, and in prostatic and breast cancer cells.[15～21] Expression of ALDH is also known to be related to tumorigenesis. In this study, expression of ALDH1A1 in CD133-positive Tosca23S cells was approximately 9-fold greater compared with the parental cells. Therefore it is conceivable that ALDH is a marker of stem cells in SCC. Furthermore, the low level of ALDH expression has been recognized in the basal cell layer of oral squamous

epithelium, but also shows proliferation activity, suggesting that ALDH may be a plausible marker for CSCs in the oral epithelium.[22, 23)]

Krt15 is known to be localized in basal cells of the squamous epithelium, and in hair follicle stem cells of the bulge region.[24~27)] In this study, Krt15 expression was approximately 6-fold higher in CD133-positive Tosca23S cells than in parental cells. In addition, strong expression of Krt15 was detected in poorly differentiated OSCC and chemo resistant OSCC, demonstrating the possibility that Krt15 is involved in cancer progression and chemo sensitivity. However, the expression of Krt15 has been previously reported to be reduced in OSCC.[28, 29)] Therefore, further study will be needed to verify the exact function of Krt15 in OSCC.

The gene SOX2 is a member of the SRY-related HMG-box family of transcription factors and has a role in embryonic development. SOX2 is expressed in gastric, pancreatic, and breast cancers.[30, 31)] Interestingly, SOX2 is one of the four transcription factors used to form human induced pluripotent stem cell (iPS cells). It has been shown that SOX2 is an indispensable gene in ES and somatic stem cells.[32, 33)] In this study, expression of SOX2 was 5-fold higher in CD133-positive Tosaca23S cells than in parental cells. In addition, it is known that transcriptional control of SOX2 is carried out by FGF3. Consistent with this, expression of FGF3 in CD133-positive Tosaca23S cells was approximately 3 times higher than that of parental cells. These results suggest that a SOX2-related pathway is activated in CD133-positive Tosca23S cells.[34)]

WNT proteins combine with Frizzled, and nuclear internal transmigration of β-catenin is stimulated. β-catenin controls the expression of cell proliferation in relation to genes such as c-myc and the cell adhesion gene. Thus, the WNT/βcatenin pathway is a common and well-known pathway involved in stem cell activity and cancer progression. The expression levels of Wnt in CD133-positive Tosaca23S cells was about 3 times higher than that in parental cells, indicating that the Wnt/βcatenin pathway may be important in regulating the function of CSCs in OSCC.

The expression of 23 genes in CD133-positive Tosaca23S cells was compared with that in parental cells. In particular, the expression of COL1A1 was about 5-fold lower than in the parental cells, and TUBB3 showed a 4-fold reduction in expression as compared with parental cells. The COL1A1 gene is expressed in many connective tissues, with its corresponding protein product a major component of the type I collagen. The exact role of COL1A1 expression in CD133-positve Tosca23S cells is unclear, but might be associated with epithelial-mesenchymal transition.

All of our results demonstrate that the expression of several stem cell markers is increased in CD133-positive Tosca23S cells as compared with parental cells, indicating that CD133 may be useful in purifying CSCs from OSCC. More detailed studies of these cells would be extremely helpful for identifying new targets to treat OSCC.

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