CD10 as a novel marker of therapeutic resistance and cancer stem cells in head and neck squamous cell carcinoma

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**Background:** Cancer stem cells (CSCs) are responsible for treatment failure. However, their identification and roles in resistance are not well established in head and neck squamous cell carcinoma (HNSCC).

**Methods:** Three HNSCC cell lines (FaDu, Detroit562 and BICR6) were treated with cisplatin or radiation. Cell surface antigens were analysed by LyoPlate, a novel cell surface antigen array. The expression levels of antigens highly expressed after treatments were further compared between cisplatin-resistant Detroit562 cells and its parental line. Association of the candidate antigen with CSCs properties, namely sphere formation and in vivo tumourigenicity, was also examined.

**Results:** CD10, CD15s, CD146 and CD282 were upregulated across the treated cell lines, while the increased expression of CD10 was prominent in the cisplatin-resistant cell line. Isolation mediated by FACS revealed that the CD10-positive subpopulation was more refractory to cisplatin, fluorouracil and radiation than the CD10-negative subpopulation. It also showed an increased ability to form spheres in vitro and tumours in vivo. Moreover, the CD10-positive subpopulation expressed the CSC marker OCT3/4 at a higher level than that in the CD10-negative subpopulation.

**Conclusions:** CD10 is associated with therapeutic resistance and CSC-like properties of HNSCC. CD10 may serve as a target molecule in the treatment of refractory HNSCC.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide (Argiris et al, 2008). Despite recent advances in its diagnosis and management, long-term survival of patients with HNSCC remains poor (Lo et al, 2003). Radiotherapy and chemotherapy initially control tumour growth; however, over time many patients suffer relapse. To improve prognosis, the establishment of a novel marker to predict therapeutic resistance is required. This would also aid the optimisation of HNSCC treatment, and thus benefit patient outcome.

Cancer stem cells (CSCs) are defined as cells that possess the properties of tumour initiation and self-renewal. It is currently understood that CSCs are responsible for treatment failure in a diversity of cancers (Bao et al, 2006; Li et al, 2008). CD44 (Prince et al, 2007) and ALDH1 (Chen et al, 2009) have been reported to represent candidate markers of HNSCC CSCs; however, whether they serve as true markers remains controversial (Chen et al, 2011; Koukourakis et al, 2012). These discrepant reports prompted us to search for a novel marker specific to HNSCC CSCs. Thus, in the present study we aimed to identify a new cell surface antigen that is

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide (Argiris et al, 2008). Despite recent advances in its diagnosis and management, long-term survival of patients with HNSCC remains poor (Lo et al, 2003). Radiotherapy and chemotherapy initially control tumour growth; however, over time many patients suffer relapse. To improve prognosis, the establishment of a novel marker to predict therapeutic resistance is required. This would also aid the optimisation of HNSCC treatment, and thus benefit patient outcome.

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involved in therapeutic resistance, and to address whether it served as a marker for HNSCC CSCs. Through array analysis and testing of cell viability in the presence of therapeutic agents, we identified CD10 as a potential marker of refractory HNSCC. Moreover, CD10 was found to confer a CSC-like phenotype, and underscored expression of OCT3/4. Thus, CD10 could be a specific marker of HNSCC CSCs that contributes to therapeutic resistance.

**MATERIALS AND METHODS**

**Cell culture.** FaDu and Detroit562 cell lines were obtained from the ATCC (Manassas, VA, USA), while BICR6 was from ECACC (Proton Down, Salisbury, UK). FaDu and BICR6 were established from a primary hypopharyngeal cancer, while Detroit562 were from a lymph node metastasis of pharyngeal cancer. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) and a penicillin (50 U ml⁻¹) and streptomycin (50 μg ml⁻¹) cocktail under an atmosphere of 5% CO² at 37 °C. The cisplatin-resistant Detroit562 cell line was established by continuous stepwise exposure to cisplatin starting from a concentration of 1 μM.

**Cell surface antigen arrays.** Cells were exposed to 3 μM cisplatin for 7 days. Alternatively, cells were irradiated by a single fraction of 8 Gy and further cultured for 5 days. The expression patterns of cell surface antigens were then compared between the treated and untreated cells using the LyoPlate cell surface antigen array (BD Biosciences, San Jose, CA, USA). The kit consists of three 96-well plates coated with monoclonal antibodies along with AlexaFluor 647 conjugated goat anti-mouse Ig and goat anti-rat Ig secondary antibodies. It allows comprehensive analysis of 242 cell surface antigens by flow cytometry, which was performed using the Cell Analyzer EC800 (Sony, Tokyo, Japan).

**Flow cytometry and cell sorting.** Flow cytometry and cell sorting were performed using the FACSaria II (BD, Franklin Lakes, NJ, USA). Cells were harvested and single-cell suspensions were prepared with the aid of StemPro Accutase (Life Technologies, Carlsbad, CA, USA). Spheroid cells were separated into single-cell suspensions with the aid of collagenase I (Sigma Aldrich) and adjusted to a concentration of 10³ cells ml⁻¹. To stain surface antigens, cells were incubated with antibodies against CD10, CD15s, CD44, CD146, and CD282 for 30 min on ice. The fluorophores for each antibody were as follows: CD10—Brilliant Violet (Biolegend, San Diego, CA, USA) and APC (BD Biosciences); CD44—FITC (BD Biosciences); CD146—APC (Biolegend); CD282—PE (BD Biosciences). For CD15s, we combined purified antibody (BD Biosciences) and the secondary antibody—APC/Cy7 (BD Biosciences). The antibodies against CD10, CD15s, and the secondary antibody of CD15s were used at a concentration of 50 μl ml⁻¹. The antibodies against CD44, CD146, and CD282 were used at a concentration of 200 μl ml⁻¹. To stain ALDH1, we used the Aldefluor stem cell detection kit (StemCell Technologies, Vancouver, BC, Canada) at a concentration of 50 μl ml⁻¹ for 45 min at 37 °C. The fluorophore of Aldefluor was FITC. Doublet cells were eliminated using FSC-A/FSC-H and SSC-A/SSC-H. Dead and damaged cells were eliminated using 7-AAD (BD Biosciences). Briefly, after CD10, CD15s, CD44, CD146, CD282 and ALDH1 staining, 7-AAD was incubated with cells for 10 min at room temperature. Except cell sorting, all FACS analysis was performed three times.

**Viability assay.** Cells were seeded in 96-well plates at 3 × 10³ cells per well, cultured overnight and then incubated with 0.1–5 μM cisplatin or 0.5–50 μM fluorouracil for 72 h. Alternatively, cells were irradiated at a single fraction of 8 Gy and then cultured for 72 h. Cell viability was subsequently measured using the Cell Counting Kit-5 (Dojindo Laboratories, Kamimasaki, Japan). The assay was performed three times.

**Sphere formation assay.** Cells were seeded in 96-well flat bottom ultra-low-attachment culture dishes (Corning, Tewksbury, MA, USA) at 10 cells per well in ReproStem medium (ReproCELL, Yokohama, Japan) containing penicillin (50 U ml⁻¹) and streptomycin (50 μg ml⁻¹) cocktail and basic fibroblast growth factor (5 ng ml⁻¹) without FBS. After 10 days, the size of spheroid colonies was measured under a microscope and the number of colonies with a diameter over 100 μm was counted. The assay was performed three times.

**Xenograft assay.** The various numbers of cells (1 × 10², 1 × 10³ and 1 × 10⁴) were diluted in equal amounts of DMEM and Matrigel (BD Biosciences) to a final volume of 200 μl then injected subcutaneously into NOD/SCID mice (Charles River Laboratories Japan, Yokohama, Japan) using a 22-gauge needle. The mice were maintained under pathogen-free conditions and sacrificed 2 months later or when tumours exceeded 20 mm at the largest diameter. Mice were handled in accordance with the procedures outlined in the Regulations on Animal Experiments at Osaka University. The institutional committee on animal research approved the study.

**Quantitative real-time PCR.** Quantitative real-time PCR was used to validate siRNA-mediated knockdown of CD10 and to examine mRNA levels of OCT3/4. Briefly, total RNA was isolated from cells using Trizol reagent (Life Technologies) and cDNA was synthesised using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative reverse transcription-PCR (qRT–PCR) was performed using a Light Cycler TaqMan Master (Roche, Basel, Switzerland). The primer sequences were as follows: CD10 5'-GGGGAGGCTTTATGTGGAGG-3' (sense) and 3'-CTC GGATCGTGTGCAATCAA-5' (antisense); and OCT3/4 5'-GAAA CCCACAGTGCAGA-3' (sense) and 3'-CGGTGACTGAGAACC ACACTCG-5' (antisense). Gene expression levels were normalised to that of ACTB, 5'-AGAGCTAGAAGCTGCCTG-3' (sense) and 3'-CGTGAGAAGGCAAGGACT-5' (antisense).

**Transfection.** The siRNA duplexes, si-CD10 and si-control, were obtained from Life Technologies. The si-CD10 sequences were as follows: 5'-GCCCUCUUAUAGGUAAACCUAGAC-3' (sense) and 3'-UUUCUAGGUUUGAUAAGGCC-5' (antisense). An initial dose-response experiment was performed according to the manufacturer’s instructions to determine optimal transfection efficiency. Optimal inhibition was observed at a concentration of 10 nM siRNA at 72 h after transfection, thus further qRT–PCR analysis was done under these conditions.

**Statistical analysis.** The comparison of spheroid colony sizes was made using the Mann–Whitney U-test. The analyses of viability curves were made using two-way analysis of variance. Other statistical comparisons were made using the Student’s t-test. Differences were considered significant when P < 0.05. All statistical analyses were performed using JMP Pro 11 (SAS Institute, Cary, NC, USA).

**RESULTS**

**Identification of antigens related to therapeutic resistance.** To identify antigens related to therapeutic resistance, surface antigen expression levels in cells from three HNSCC cell lines, Detroit562, FaDu and BICR6 that survived treatment with cisplatin or radiation were compared with those of their untreated counterparts by means of LyoPlate (Supplementary Table 1). From this analysis, four cell surface antigens, CD10, CD15s, CD146 and CD282 were identified as potential markers of refractory HNSCC. Through array analysis and testing of cell viability in the presence of therapeutic agents, we identified CD10 as a potential marker of refractory HNSCC. Moreover, CD10 was found to confer a CSC-like phenotype, and underscored expression of OCT3/4. Thus, CD10 could be a specific marker of HNSCC CSCs that contributes to therapeutic resistance.
CD10 and sphere formation ability. Given that CSCs are responsible for therapeutic resistance (Bao et al, 2006; Li et al, 2008), and are also in the dormant or slow-growing phase of the cell cycle (Holyoke et al, 1999), we hypothesised that CD10 might be a novel marker for CSCs in HNSCC. One of the most important characteristics of CSCs is self-renewal ability, which is assessed by sphere formation. First, we examined the distribution of CD10 in spheroid cells and control adherent cells using FACS analysis. In FaDu, 10.3% of spheroid cells and 2.1% of adherent cells were CD10(+) (Figure 3A and B). Next, we compared sphere formation ability between CD10(+) and CD10(−) subpopulations. Although the morphology of spheroid colonies was similar between the two subpopulations (Figure 3C), there was a significant difference in their number. The CD10(+) subpopulation formed more spheroid colonies than the CD10(−) subpopulation in both FaDu and Detroit562 (Figure 3D–E). Moreover, colonies of the CD10(+) subpopulation were larger than those of the CD10(−) subpopulation in FaDu and Detroit562 (Figure 3F–G).

CD10 and tumourigenicity. To further address the association between CD10 and CSC properties, we examined whether CD10 modulates in vivo tumourigenicity. CD10(+) and CD10(−) subpopulations were sorted and individually transplanted into NOD/SCID mice. The result of the limiting dilution transplantation assay of Detroit562 cells is shown in Table 2. Briefly, when 1000 cells were transplanted, the CD10(+) subpopulation formed tumours in six out of 100 (6%) transplanted mice, while the CD10(−) subpopulation formed tumours in only two out of six (33%) mice. Moreover, the CD10(+) subpopulation remained tumorigenic with as few as 100 cells. In contrast, there was no difference in tumourigenicity between the CD10(+) and CD10(−) subpopulations of FaDu (Supplementary Table 2), although the size of tumours formed by inoculation of 1000 cells was notably larger in the CD10(+) subpopulation than in the CD10(−) subpopulation (Supplementary Figure 1). To confirm that the histology of tumours was squamous cell carcinoma, we performed H&E staining (Figure 4A). Both FaDu and Detroit562 tumours from CD10(+) and CD10(−) subpopulations presented with squamous cell carcinoma histology and the shapes of these tumour cells were similar to those of parental cell lines.

Interrelations between CD10 and other CSC markers. It has been reported that CD44 (Prince et al, 2007), CD133 (Chiou et al, 2008) and ALDH1 (Chen et al, 2009) are markers of CSCs in HNSCC; thus, we examined the interdependence between CD10 and these markers. Since CD133 is not contained in the cell surface antigen array, we first assessed its expression in treated (cisplatin or radiation) and untreated FaDu cells by flow cytometry as per the conditions used in the array analysis. We found that CD133 expression was barely detectable even after the treatments

| Cell surface antigen | Control (%) | RT (%) | CDDP (%) | Control (%) | RT (%) | CDDP (%) | Control (%) | RT (%) | CDDP (%) |
|----------------------|-------------|--------|----------|-------------|--------|----------|-------------|--------|----------|
| CD10                 | 7.5         | 36.5   | 31.8     | 9.5         | 23.1   | 24.3     | 31.2        | 38.1   | 57.6     |
| CD15s                | 40.7        | 52.6   | 66.8     | 25.5        | 95.6   | 74.2     | 58.4        | 65.5   | 78.3     |
| CD146                | 64.5        | 76.8   | 89.2     | 19.6        | 82.9   | 37.3     | 3.7         | 9.2    | 15.2     |
| CD282                | 8.6         | 26.7   | 27.6     | 11.5        | 98.8   | 14.3     | 7.1         | 25.3   | 30.3     |

Abbreviations: CDDP, cisplatin; RT, radiotherapy. Control, no treatment; RT, cells were assayed 5 days after exposure to single fraction 8 Gy irradiation; CDDP, cells were assayed after exposure to 3 μM cisplatin for 7 days. Data represent the percentages of each marker as measured by flow cytometry.
Thus a relationship between CD10 and CD133 by means of FACS analysis could not be explored. As for CD44, we found that the majority of FaDu and Detroit562 cells were CD44(+) (Supplementary Figure 2A), although we found that all CD10(+) cells expressed CD44 in both Detroit562 and FaDu cell lines. Significant interdependence was not detected. As for ALDH1, we found that CD10(+) cells expressed significantly more ALDH1 than CD10(-) counterparts in both cell lines (Figure 4B and Supplementary Figure 2B). The expression levels of CD10 and ALDH1 were found to be interdependent.

Stem cell-related genes in CD10-positive cells. To shed light on the molecular mechanisms underlying self-renewal ability and tumourigenicity of the CD10(+) subpopulation, we compared the expression of OCT3/4, a known marker of tissue stem cells (Nichols et al, 1998) and CSCs (Nichols et al, 1998), between CD10(+) and CD10(-) subpopulations. OCT3/4 expression was significantly increased in the CD10(+) subpopulation when compared with that of the CD10(-) subpopulation in both FaDu and Detroit562 (Figure 4C). Of note, knockdown of CD10 by siRNA resulted in decreased expression of OCT3/4 (Figure 4D and Supplementary Figure 3A–B).

**DISCUSSION**

In the present study, we used the novel cell surface antigens array Lyoplate to identify antigens relevant to cell survival after...
treatment with cisplatin or radiation. This is the first report that tries to identify an antigen that exhibits both therapeutic resistance and is related to CSCs by means of the cell surface antigens array. We found that CD10, CD15s, CD146 and CD282 were highly expressed in treated cells compared with untreated cells. To validate the result of the cell surface antigens array, we next compared the expression of these antigens between a cisplatin-resistant cell line and its parental cell line. Of the candidate antigens, only expression of CD10 was upregulated in the cisplatin-resistant cell line as determined by FACS analysis. We propose two reasons for the different antigen expression profiles detected by Lyoplate and FACS analysis. First, different flow cytometers were used for the detection of signals, thus variations in sensitivity may account for the divergent findings.
Second, it is the difference of products of antibodies such as clone number, type of fluorophores and method of staining. These may further underlie differences in technical sensitivity. However, both techniques clearly demonstrated that CD10 was upregulated in response to either cisplatin or radiation treatment, as well as in the cisplatin-resistant cell line.

CD10, also known as membrane metalloendopeptidase, neutral endopeptidase, neprilysin and common acute lymphoblastic leukaemia antigen (CALLA), is a zinc-dependent
metalloendoprotease that cleaves signalling peptides (Roques et al., 1993; Turner & Tanzawa, 1997). It is expressed in a wide range of normal cells, and has been shown to be a cell surface marker of tissue stem cells in the bone marrow (Galy et al., 1998), adipose (Buhring et al., 2007), lung (Sunday et al., 1992) and breast (Stingl et al., 2005). CD10 is also expressed in a series of malignancies originating from the kidney, lung, skin, pancreas, prostate, liver, breast, stomach, cervix and bladder. Several studies have shown an association between CD10 and metastasis (Maguer-Satta et al., 2011). In HNSCC, an involvement of CD10 in tumour differentiation and growth has been reported (Piattelli et al., 2006). This report also showed that expression of CD10 was associated with distinct metastases, local recurrences and histological grade in HNSCC patients. Because of this background and our experiments, we hypothesised that CD10 is a marker for refractory HNSCC. Thus, we further examined whether the CD10-positive subpopulation was chemo and/or radio resistant. We found that the CD10-positive subpopulation was more resistant to treatment with cisplatin, fluorouracil or radiation in comparison with the CD10-negative subpopulation. Several mechanisms, such as efficient DNA repair and expression of transporter pumps, as well as changes in cell cycling are considered to explain such resistance. Among these mechanisms, we focussed on the cell cycle. We analysed cell cycle phase distributions between CD10-positive and CD10-negative subpopulations. We found that the percentage of G0/G1 phase cells was increased in CD10-positive subpopulation when compared with that of the CD10-negative subpopulation. This result indicates that the CD10-positive subpopulation of HNSCC cells was slow-cell cycling or dormant compared with CD10-negative subpopulation.

Recent studies have shown that CSCs are responsible for the therapeutic resistance of cancers (Bao et al., 2006; Li et al., 2008).

Figure 4. Histology of tumours from CD10(+) or CD10(−) subpopulations and the relationship between CD10 and other stem cell markers. (A) H&E staining of FaDu and Detroit562 xenograft tumours. Scale bar, 100 μm. (B) Expression of ALDH1 in CD10(+) or CD10(−) FaDu and Detroit562 cells was assessed by FACS. Data represent means ± s.e.m.; **P<0.01. (C) OCT3/4 expression in CD10(+) or CD10(−) FaDu and Detroit562 cells was assessed by qRT-PCR. (D) OCT3/4 expression in FaDu and Detroit562 following transfection with either si-CD10 or si-control was assessed by qRT-PCR. Gene expression levels are presented as a ratio of the internal control, ACTB ± s.e.m. *P<0.05; **P<0.01.
Additionally, CSCs are slow-cycling or in the dormant phase of the cell cycle. For example, CSCs of acute myeloid leukaemia (Guan et al, 2003) and chronic myeloid leukaemia (Holyoake et al, 1999) survive in the dormant G0 phase of the cell cycle. In the case of solid tumours, liver CSCs were found to be mainly in the G0/G1 phase (Haraguchi et al, 2010). Thus, we addressed the relevance of CD10 for the CSC phenotype. We found that the CD10-positive subpopulation formed spheres in vitro and tumours in vivo more efficiently than the CD10-negative subpopulation. These results indicate that CD10 is closely related to tumourigenicity and self-renewal ability. Thus, it seems likely that CD10 could serve as a marker of CSCs in HNSCC.

Previously, CD44 (Prince et al, 2007), CD133 (Chen et al, 2008) and ALDH1 (Chen et al, 2009) have been reported as markers of CSCs in HNSCC. However, whether CD44 and ALDH1 serve as true markers remains controversial. For instance, recent studies have shown that decreased rather than increased expression of ALDH1 is linked to poor prognosis (Koukourakis et al, 2008) and ALDH1 (Chen et al, 2009) is associated with refractory HNSCC. For example, CSCs of acute myeloid leukaemia (Guan et al, 1999) are slow-cycling or in the dormant phase of the cell cycle. Thus, we considered the combination of CD10 and ALDH1, because we found these interdependent expressions.

Notably, we demonstrated that the CD10-positive subpopulation of HNSCC cells showed CSC-related properties, such as chemo and radio resistance, self-renewal capacity and tumourigenicity. To gain insight into the mechanisms by which CD10 confers CSC-related properties in HNSCC, we examined the expression of OCT3/4, which has a critical role in the development and self-renewal of embryonic stem cells (Nichols et al, 1998). It is linked to oncogenic processes (Gidekel et al, 1999). OCT3/4 is upregulated in HNSCC CSCs, defined by ALDH1 positive cells, and in spheroid forming HNSCC cells. We found that OCT3/4 expression was higher in CD10-positive cells than in CD10-negative cells, but that it was decreased following knockdown of CD10. These results indicate that increased CD10 is linked to OCT3/4 expression. Further studies are required to address the functional relevance of CD10 to OCT3/4 in HNSCC.

In conclusion, we have established that CD10 is associated with chemo and radio resistance, and that it confers CSC-related properties in HNSCC, probably through forced overexpression of OCT3/4. Together these findings suggest that CD10 may serve as a target molecule in the treatment of refractory HNSCC.

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