

Analysis of immunoexpression of common cancer stem cell markers in ameloblastoma

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Received October 5, 2011; Accepted December 2, 2011

DOI: 10.3892/etm.2011.437

Abstract. Recent studies have established that, in benign tumors, a large number of cancer stem cells are present, which have great implications in tumor development. However, in ameloblastoma, a highly aggressive, locally invasive tumor with a high recurrence rate, whether or not cancer stem-like cells are present remains undetermined. Therefore, in this study we analyzed the protein expression of three candidate stem cell markers in ameloblastoma. Immunohistochemical staining for cancer stem cell (CSC) markers (CD133, CD44 and ABCG2) and for the proliferation marker Ki-67 was performed using 23 ameloblastoma cases. In all 23 samples, CD133, CD44 and ABCG2 were expressed. Nine (39.13%) cases showed high expression and 14 cases (60.87%) showed low expression for CD133. Twelve of the 23 cases (52.17%) showed high expression and 11 cases (47.83%) showed low expression for both CD44 and ABCG2, respectively. Ki-67 was mainly expressed in peripheral ameloblast-like cells, suggesting that these cells have a higher degree of differentiation and, therefore, are less likely to contain cancer stem-like cells. On the other hand, cells positive for CSC markers situated at the close proximity to peripheral cells were devoid of Ki-67 and may have the potential to be cancer stem-like cells. After analyzing the correlation between expression of three CSC markers with clinicopathological factors and Ki-67 expression, only CD44 expression was correlated with tumor recurrence ($P=0.0391$). In conclusion, this study showed various expression patterns of different types of cancer stem cell markers and the presence of candidate CSC-like cells in ameloblastoma, which are possibly involved in cell proliferation, tumor progression and recurrence.

Introduction

Ameloblastoma is the most frequently encountered benign, locally invasive tumor and the second most common odontogenic tumor (1). It may arise from rests of dental lamina, from a developing enamel organ or from basal cells of the oral mucosa. Ameloblastomas tend to infiltrate between intact cancellous bones at the periphery of the lesion before bone resorption becomes radiographically evident. As a result, the actual margin of the tumor often extends beyond its apparent radiographic or clinical margin. Attempts to remove the tumor often leave small islands of tumor, which later result in recurrence in 50-90% of cases (2). This has raised questions regarding the tumor cell populations that are responsible for tumor growth and recurrence.

In the past few years, it has been hypothesized that tumors are most likely initiated in normal stem cells or their immediate descendants, and then are perpetuated by a minority of these cells, known as cancer stem cells (CSCs) or tumor-initiating cells (TICs) (3). According to the American Association for Cancer Research (AACR), 'a cell within a tumor that possesses the capacity to self-renew and to cause heterogeneous lineages of cancer cells that comprise the tumor is known as a cancer stem cell'. This observation implies that within a given tumor, there exists a small population of cells with the capacity to behave like stem cells (4). The difficulty in eradicating tumors may be due to the fact that conventional treatments target the bulk of the tumor cells leaving unaffected the CSCs, which like their normal counterparts, maintain tumor tissue. According to this hypothesis, identifying and exterminating CSCs may be an effective treatment modality (5).

Recently, several CD markers have been identified as solid CSC markers. CD133, also known as PROM1 or prominin, is a stem cell surface antigen that has been recently identified as a potential CSC marker in brain, colon, hepatocellular and prostate cancer (6-10). CD44, also known as homing cell adhesion molecule, is a cell surface glycoprotein expressed on lymphocytes, monocytes and granulocytes, which has been identified as a stem cell marker for breast, prostate, pancreatic and head and neck cancer (6,11-13). The ABCG2 transporter is a member of the ATP binding cassette transporter family (14) responsible for the side population phenotype in various

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Key words: ameloblastoma, cancer stem cell, CD133, CD44, ABCG2, immunohistochemistry

Table I. Clinical and histopathological data of the patients with ameloblastoma.

Case no.	Gender	Age (years)	Location	Histological type
1	Female	41	Mandible	Solid multilocular (Plexiform)
2	Male	60	Mandible	Solid multilocular (Mixed)
3	Male	58	Mandible	Solid multilocular (Mixed)
4	Male	31	Mandible	Solid multilocular (Plexiform) ^a
5	Male	69	Mandible	Solid multilocular (Mixed)
6	Male	71	Mandible	Solid multilocular (Mixed) ^a
7	Male	39	Maxilla	Solid multilocular (Mixed) ^a
8	Male	25	Maxilla	Solid multilocular (Plexiform)
9	Male	25	Maxilla	Solid multilocular (Mixed) ^a
10	Male	21	Maxilla	Solid multilocular (Mixed)
11	Female	46	Mandible	Solid multilocular (Mixed) ^a
12	Female	42	Mandible	Solid multilocular (Mixed)
13	Female	30	Maxilla	Solid multilocular (Plexiform) ^a
14	Female	27	Maxilla	Solid multilocular (Plexiform)
15	Male	45	Mandible	Solid multilocular (Follicular) ^a
16	Male	44	Maxilla	Solid multilocular (Mixed)
17	Male	50	Mandible	Solid multilocular (Follicular)
18	Male	53	Mandible	Solid multilocular (Mixed) ^a
19	Male	55	Mandible	Solid multilocular (Mixed) ^a
20	Male	55	Mandible	Solid multilocular (Mixed) ^a
21	Female	83	Maxilla	Solid multilocular (Plexiform) ^a
22	Female	81	Maxilla	Solid multilocular (Mixed)
23	Female	37	Mandible	Solid multilocular (Follicular)

^aRecurrent case.

human cancers and the corresponding non-malignant tissues, and is widely used to detect and isolate somatic stem/progenitor cells (7). Xu *et al* for the first time demonstrated that stem-like cells are present in benign tumors. They isolated self-renewable and multipotent stem-like cells from various pituitary adenomas. Implanted into immune compromised mice, these cells initiated transplantable pituitary tumors that resembled the primary tumors (15).

Ameloblastoma is a benign tumor with two histologically distinct cell types: peripheral ameloblast-like cells and central stellate reticulum-like cells. The presence of cancer stem-like cells in ameloblastoma remains undetermined. However, if cancer stem-like cells are present in ameloblastoma, it is important to identify which type of cell possesses these cancer stem-like characteristics and is responsible for ameloblastoma progression and recurrence. Therefore, in this study we analyzed the protein expression of the three most putative candidate stem cell markers, CD133, CD44 and ABCG2, in ameloblastoma.

Materials and methods

Tissue sample selection. Twenty-three blocks from 17 cases embedded in paraffin were selected from the Surgical Pathology Unit of the Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and

Pharmaceutical Science of Okayama University, Japan. These samples were fixed in 10% neutral buffered formalin and routinely processed and embedded in paraffin. Histological diagnosis was carried out by routine H&E, according to WHO histological typing of odontogenic tumors. Clinical characteristics and histological types of the cases are shown in Table I.

Immunohistochemistry. Sections (3- μ m) mounted on salinized slides were used for immunohistochemical staining. Briefly, sections were deparaffinized in a series of xylene for 15 min and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H₂O₂ in methanol for 30 min. Antigen retrieval was achieved by heat treatment using 10 mM citrate buffer solution pH 6.0 (CD133, CD44, ABCG2 and Ki-67). After treatment with normal serum, the sections with primary antibodies were incubated at 4°C overnight. The tagging of the primary antibody was achieved by subsequent application of anti-goat/mouse IgG and avidin-biotin complexes (Mouse ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) or Envision peroxidase detecting reagent (Dako, Carpinteria, CA, USA). Visualization of the immunohistochemical reaction was performed by developing the enzyme complex with DAB/H₂O₂ solution (Histofine DAB substrate; Nichirei, Japan) and counterstaining with Mayer's hematoxylin. The antibodies used are listed in Table II.

Table II. Details of the antibodies used.

Antibody	Clonality	Supplier	Dilution	Treatment
CD133	Rabbit polyclonal	Abcam, Cambridge, MA, USA	1:300	Heat
CD44	Rabbit polyclonal	Abcam, Cambridge, MA, USA	1:200	Heat
ABCG2	Mouse monoclonal	Abcam, Cambridge, MA, USA	1:50	Heat
Ki-67	Mouse monoclonal	Dako, Denmark	1:50	Heat

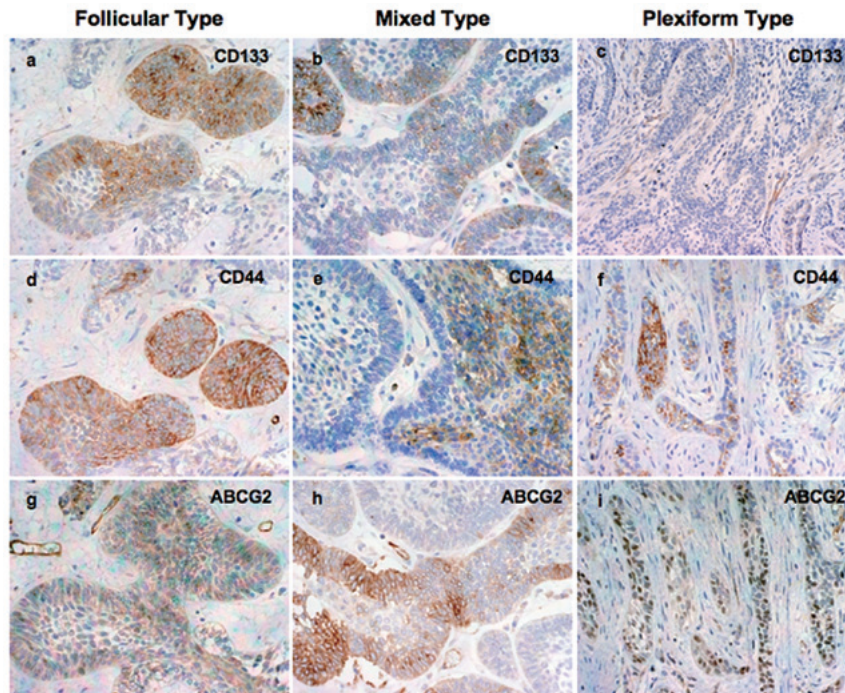


Figure 1. Immunostaining for (a-c) CD133, (d-f) CD44 and (g-i) ABCG2 in different types of ameloblastoma.

Ki-67-positive cell count. For analysis of the percentage of Ki-67-positive cells, in each tissue section, several fields were chosen randomly and the fields that complied with the requirements were further selected. The requirements were that the well-preserved fields were stained by anti-Ki-67 antibodies. Clear brown nuclei were regarded as positive cells, and at least three to five fields in each section were counted using an eyepiece micrometer and counted at x40 magnification. The process was repeated three times to decrease the operator error. The percent labeling index (LI) (Number of positive cells/total cells x 100) was calculated for each field.

Scoring for protein expression. Immunoreactivity with each of the antibodies was graded in each histopathological compartment of ameloblastoma: low expression (0-30%) and high expression ($\geq 31\%$).

Statistical analysis. The LI of Ki-67 was counted; the mean and the standard deviation (SD) were calculated. The Student's t-test was used, and P-values < 0.05 were considered to denote statistical significance. Clinicopathological data and low and high expression of different CSC markers were statistically compared using the t-test and Fisher's exact test, and P-values < 0.05 were considered to denote statistical significance.

All computations were computer-based using Stat View for Windows version 5.0 statistical program, SAS Inc.

Results

Immunohistochemistry. Representative immunohistochemical staining for the three CSC proteins investigated in this study are shown in Fig. 1.

CD133. The location of staining was cytoplasmic. Intense immunostaining was observed in small tumor follicles and peripheral ameloblast-like cells. In plexiform type, several cases showed a positive immunoreaction with peripheral columnar cells, with few central cells at the close vicinity of the peripheral ameloblast. Certain cases showed a weak or negative reaction. Nine (39.13%) cases showed high and 14 cases (60.87%) showed low CD133 expression (Fig. 1a-c).

CD44. The location of staining was cytoplasmic. In several cases, CD44 expression was strong in small tumor follicles. However, plexiform type ameloblastoma showed intense immunoreaction in center stellate reticulum-like cells. Twelve of 23 cases (52.17%) showed high and 11 cases (47.83%) showed low CD44 expression (Fig. 1d-f).

ABCG2. The location of staining of these molecules was also cytoplasmic. Diffuse and intense staining was observed in peripheral ameloblast-like cells, the budding area and central stellate reticulum-like cells of the tumors. Small tumor follicles showed weak immunorexpression. High ABCG2 expression was observed in 12 (52.17%) out of 23 cases and 11 cases (47.83%) showed low expression (Fig. 1g-i).

Ki-67 expression and LI. Ki-67 antigen was expressed in 82.6% of ameloblastoma cases and the positive cells present in the different histological types were counted. Ki-67 expression was mainly observed in the nuclei of the peripheral ameloblast-like cells, and only a few positive cells were observed in the central stellate reticulum-like cells (Fig. 2). Most of the tissue sections did not contain positive central cells, and peripheral cells are known to reflect the growth activity of ameloblastoma (16). The mean and standard deviation of Ki-67 LI was 23.13 and 13.9, respectively.

Correlation between CSC expression and clinicopathological factors. As shown in Table III, only CD44 expression was correlated with tumor recurrence (0.0391) and ABCG2 was correlated with the tumor location (0.0361). However, there was no significant association between CD133 expression and clinicopathological features.

Correlation between CSC expression and Ki-67-positive cells. Next, we analyzed the possible correlation between the high and low expression of three CSC markers and Ki-67 expression in all ameloblastoma cases using the Student's t-test. No significant association was observed between Ki-67 expression and expression of all three CSC markers (Table III).

Discussion

Tumor recurrence after curative surgery remains a major obstacle for improving overall cancer survival. Recurrence may be in part due to the existence of CSCs. Growing evidence suggests that human cancers are stem cell diseases, and only a small subpopulation of cancer cells, endowed with stem cell-like features, may be responsible for tumor initiation and progression (17). Novel strategies for successful tumor treatment should focus on the elimination of CSCs. In this study, we analyzed immunohistochemical staining to detect the expression of three different CSC markers in relation to the proliferation activity of the tumor cells in ameloblastoma, a highly apoptosis-resistant and invasive tumor with a high recurrence rate and possessing different histological patterns (18-21).

Increasing evidence highlights the role of CD133 as a marker of CSCs in various human tumors as well as in ameloblastoma (22,23). CD44 is one of the cell surface markers currently used to identify CSCs in various solid tumors (6,11,12). CD133 was found to be expressed in combination with CD44⁺ in prostate tumors. These cells were found to be capable of self-renewal, proliferation and multi-lineage differentiation *in vitro* to recapitulate the original tumor phenotype, consistent with CSC properties (6). In the present study, CD133, the most important CSC marker, was stained fairly well in 39.13% of the cases. CD44 is another important candidate stem cell marker and demonstrated positive expression in 52.17% of the cases. The

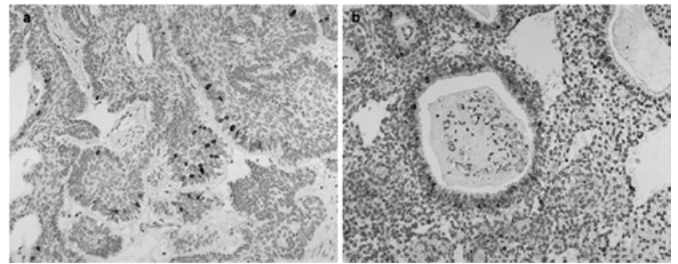


Figure 2. Representative cases of Ki-67 immunorexpression in ameloblastoma. (a and b) Ki-67 expression mainly observed in the nuclei of the peripheral ameloblast-like cells and (b) only a few positive cells were seen in the central stellate reticulum-like cells.

samples positive for both CD133 and CD44 showed that the two markers present a similar expression pattern and are mainly located in peripheral cells. This co-expression suggests that these two molecules may be potential biomarkers for the initiation, progression and cell differentiation in ameloblastoma (24).

It has already been reported that CD44⁺ cells have the capability to initiate tumor recurrence upon completion of treatment (25). In the present study, statistical data revealed that CD44 expression was significantly associated with tumor recurrence. This suggests that, in ameloblastoma, CD44 may play a central role in tumor recurrence. ABCG2 is a member of the ATP binding cassette transporter family, is widely expressed in stem cells, and is recognized as a universal marker of stem cells. Recent studies strongly suggest that ABCG2 expression in tumors may contribute to tumor growth initiation, invasiveness and relapse (26-30). In this study, diffuse expression of ABCG2 was observed in ameloblastoma, suggesting that ABCG2 may enhance the proliferation and invasion of tumors by maintaining the existing cancer stem-like cells in ameloblastoma. This also supports other studies regarding ABCG2 expression in ameloblastoma (22).

In ameloblastoma, even though different types of histological variations are present, two distinct cell types are observed in all subtypes: peripheral columnar epithelium or ameloblast-like cells and central stellate reticulum-like cells (1). Peripheral cells are always situated at the invasive front, but no morphological differentiation can be observed. By contrast, in many cases, central stellate reticulum-like cells show cellular differentiation and morphological change (1). In the present experiment, it was observed that most of the peripheral cells positive for CSC markers were also positive for the proliferative marker Ki-67. However, cell proliferation marker Ki-67-positive cells have a higher degree of differentiation and are, therefore, less likely to contain cancer stem-like cells (31). Therefore, in these cells, it is possible that CSC markers only maintain cellular proliferation and tumor progression. In contrast, few CSC marker-positive central stellate reticulum-like cells situated at the close vicinity of the peripheral cells were devoid of Ki-67 expression. Thus, these cells may have the potential to be cancer stem-like cells. In accordance with this hypothesis, these central stellate reticulum-like cells may change their morphology and differentiate into different types of cellular patterns, for example, granular, squamous and acanthomatous type (1).

The presence of candidate CSCs in ameloblastoma supports the findings of a previous study which revealed that it

Table III. Correlation between CSC markers expression and clinicopathological factors in ameloblastoma.

	CD133 expression			P-value
	Total [n=23 (%)]	High (n=9; 39.13%)	Low (n=14; 60.87%)	
Age (years; mean \pm SD) ^a	47.33 \pm 9.73	43.71 \pm 21.83	0.1874	
Gender ^b				0.0858
Male	15 (65.22)	8 (53.33)	7 (46.67)	
Female	8 (34.78)	1 (12.5)	7 (87.5)	
Location ^b				0.2283
Mandible	14 (60.87)	7 (50.0)	7 (50.0)	
Maxilla	9 (39.13)	2 (22.22)	7 (77.78)	
Recurrence ^b				0.6802
Yes	11 (47.83)	5 (45.45)	6 (54.55)	
No	12 (52.17)	4 (33.33)	8 (66.67)	
Ki-67 (mean \pm SD) ^a	27.80 \pm 13.84	19.63 \pm 13.44	0.1892	
	CD44 expression			P-value
	Total [n=23 (%)]	High (n=12; 52.17%)	Low (n=11; 47.83%)	
Age (years; mean \pm SD) ^a	48.58 \pm 16.31	41.36 \pm 19.48	0.1988	
Gender ^b				0.2203
Male	12 (52.17)	8 (66.67)	4 (33.33)	
Female	11 (47.83)	4 (36.36)	7 (63.64)	
Location ^b				0.0995
Mandible	13 (56.52)	9 (69.23)	4 (30.77)	
Maxilla	10 (43.48)	3 (30.0)	7 (70.0)	
Recurrence ^b				0.0391 ^c
Yes	11 (47.83)	3 (27.27)	8 (72.73)	
No	12 (52.17)	9 (75.0)	3 (25.0)	
Ki-67 (mean \pm SD) ^a	22.21 \pm 15.35	24.15 \pm 12.86	0.7579	
	ABCG2 expression			P-value
	Total [n=23 (%)]	High (n=12; 52.17%)	Low (n=11; 47.83%)	
Age (years; mean \pm SD) ^a	44.00 \pm 14.23	46.17 \pm 21.26	0.9039	
Gender ^b				0.1930
Male	16 (69.57)	10 (62.5)	6 (37.5)	
Female	7 (30.43)	2 (28.57)	5 (71.43)	
Location ^b				0.0361 ^c
Mandible	14 (60.87)	10 (71.43)	4 (28.57)	
Maxilla	9 (39.13)	2 (22.22)	7 (77.78)	
Recurrence ^b				0.6843
Yes	11 (47.83)	5 (45.45)	6 (54.55)	
No	12 (52.17)	7 (58.33)	5 (41.67)	
Ki-67 (mean \pm SD) ^a	25.83 \pm 15.67	21.10 \pm 12.74	0.4543	

^aStudent's t-test; ^bFisher's exact test. ^cP<0.05.

is possible that CSCs are present not only in malignant lesions, but also in benign lesions (15).

In conclusion, immunohistochemical results indicate that all three candidate CSC markers are expressed in ameloblastoma and are possibly involved in cell proliferation, tumor

progression and recurrence. Among the CSC marker-positive cells, central stellate reticulum-like cells, situated at the close vicinity of the peripheral ameloblast-like cells, may be candidate cancer stem-like cells in ameloblastoma. Understanding the biological function of the expression of CSC markers in

ameloblastoma may aid in elucidating their role in tumor pathogenesis, and continued research may lead to the development of more effective therapeutic approaches.

Acknowledgements

This study was supported by a Grant-in-Aid for Research (C) (no. 21592326) from the Japan Society for the Promotion of Science (JSPS), and a Grant-in-Aid for Young Scientists (B) (nos. 22791977, 20791337 and 22791766) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

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