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ORIGINAL PAPER

Expression status of Zic family member 2 as a prognostic marker for oral squamous cell carcinoma

Kentaro Sakuma · Atsushi Kasamatsu · Masanobu Yamatoji · Yukio Yamano · Kazuaki Fushimi · Manabu Iyoda · Kenji Ogoshi · Keiji Shinozuka · Katsunori Ogawara · Masashi Shiiba · Hideki Tanzawa · Katsuhiro Uzawa

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Abstract
Purpose To determine the involvement of ZIC2 in oral squamous cell carcinoma (OSCC).
Methods ZIC2 mRNA and protein expression in primary OSCCs ($n = 74$), oral premalignant lesions (OPLs, $n = 20$) and five OSCC-derived cell lines (HSC-2, HSC-3, OK-92, H1, and Sa3) were analyzed by quantitative reverse transcriptase-polymerase chain reaction, Western blot and immunohistochemistry (IHC). In addition, we evaluated the correlation between ZIC2 IHC scores in OSCCs and the clinicopathologic status.
Results Significant up-regulation of ZIC2 was detected in OSCC-derived cell lines ($P < 0.05$), primary OSCCs ($P < 0.05$) and OPLs ($P < 0.05$) compared with normal counterparts. Among the clinical variables analyzed, ZIC2 expression was associated with the histopathologic types of OSCC. Furthermore, the survival rates differed significantly between ZIC2-positive cases and ZIC2-negative cases.
Conclusions These results suggested that ZIC2 expression is correlated with the differentiation type of OSCC and diagnosis and might be a potential prognostic indicator and therapeutic target for OSCCs.

K. Sakuma · A. Kasamatsu · M. Yamatoji · Y. Yamano · K. Fushimi · M. Iyoda · K. Ogoshi · K. Shinozuka · H. Tanzawa · K. Uzawa (✉)
Department of Clinical Molecular Biology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan
e-mail: uzawak@faculty.chiba-u.jp

K. Ogawara · M. Shiiba · H. Tanzawa · K. Uzawa
Division of Dentistry and Oral-Maxillofacial Surgery, Chiba University Hospital, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Keywords Oral squamous cell carcinoma · Zic family member 2 · Oral premalignant lesion · qRT-PCR · Immunohistochemistry

Introduction

Oral squamous cell carcinomas (OSCCs), the most common cancer of the head and neck, accounts for over 300,000 new cancer cases worldwide annually (Lippman et al. 2005). In addition, the 5-year survival rate for OSCC remains about 50% (Okamoto et al. 2002; Weinberg and Estefan 2002). A number of etiologic factors have been implicated in the development of OSCCs, such as the use of tobacco, alcohol, or the presence of incompatible prosthetic materials (Mashberg et al. 1993; Fearon and Vogelstein 1990; Macfarlane et al. 1995). However, some patients develop OSCC without risk factors, suggesting that host susceptibility might play an important role. Therefore, we have continued efforts to discover suitable biomarkers for early diagnosis of OSCCs and to understand the disease pathogenesis as a first step toward improving treatment for OSCCs.

Microarray technologies have been helpful to analyze changes in thousands of genes and identify significant patterns. We previously reported the gene expression profiling of OSCC to identify genes associated with oral carcinogenesis (Yamano et al. 2008). Among the genes identified using microarray analysis, *ZIC2* expression was found to be significantly up-regulated.

The *ZIC* genes have encoded zinc-finger transcription factors and are comprised of five Cys2His2 zinc-finger domains (Grinberg and Millen 2005; Nagai et al. 1997; Mizugishi et al. 2004; Yang et al. 2000). Among these families, *ZIC2* regulates the tissue-specific expression of

dopamine receptor D1, the G-protein coupled receptor, which is the most abundant dopamine receptor in the central nervous system (Yang et al. 2000). Dopamine receptor D1 stimulates adenylyl cyclase and cyclic AMP-dependent protein kinases and regulates neuronal growth, development, and dopamine receptor D2-mediated events (Missale et al. 1998). Up-regulated ZIC2 has been reported in invasive human tumors, such as lung cancers, endometrial cancers, pediatric medulloblastoma and synovial sarcoma (Gure et al. 2000; Bidus et al. 2006; Pfister et al. 2007; Fernebro et al. 2006). In addition, ZIC2 antibody was isolated in sera obtained from patients with small cell lung cancer (Gure et al. 2000; Sabater et al. 2008; Vural et al. 2005).

In the current study, ZIC2 expression increased in OSCCs and oral premalignant lesions (OPLs) compared with normal oral tissues. Based on these data, we propose that ZIC2 might be a diagnostic marker and a key regulator of prognosis in OSCCs.

Materials and methods

Tissue specimens and OSCC-derived cell lines

OSCCs, OPLs and patient-matched normal oral epithelium were obtained during surgical resection at Chiba University Hospital after the patients provided informed consent for use of a protocol reviewed and approved by the institutional review board of Chiba University. The resected tissues were divided into two parts, one of which was frozen immediately and stored at –80°C until RNA isolation, and the second of which was fixed in 10% buffered formaldehyde solution for pathologic diagnosis and immunohistochemistry (IHC). Histopathologic diagnosis of each neoplastic tissue was performed according to the World Health Organization criteria by the Department of Pathology, Chiba University Hospital. Clinicopathologic staging was determined by the TNM classification of the International Union against Cancer. All patients had SCC that was histologically confirmed, and tumor samples were checked to ensure that tumor tissue was present in more than 80% of the specimens.

HSC-2 and HSC-3 cell lines, derived from human OSCCs, were purchased from the Human Science Research Resources Bank (Osaka, Japan). The OK92 cell line was established in our department. H1 and Sa3 cell lines were kindly provided from Dr. Fujita at Wakayama Medical University (Wakayama, Japan). Primary cultured human normal oral keratinocytes (HNOKs) were used as a normal control (Kasamatsu et al. 2005, Endo et al. 2004). All cells were grown in Dulbecco's modified Eagle medium/F-12 HAM (Sigma–Aldrich Co, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) and 50 units/ml penicillin and streptomycin (Sigma).

Isolation of RNA

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted RNA samples were stored separately at –80°C until use.

Preparation of cDNA

cDNA was generated using 5 μg of total RNAs from OSCC-derived cell lines, using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, UK) and oligo (dT) primer (Sigma Genosys, Ishikari, Japan), according to the manufacturer's instructions.

mRNA expression analysis

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to evaluate the expression level of ZIC2 mRNA in the OSCC-derived cell lines (HSC-2, HSC-3, OK-92, H1, and Sa3) and HNOKs. In addition, primary tumors and paired specimens of normal oral tissues from 74 patients also were evaluated. qRT-PCR was performed with a single method using a LightCycler FastStart DNA Master SYBR Green 1 Kit (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences used to analyze ZIC2 mRNA expression were forward 5′-CTCCCTGCTAATCTCCATGC-3′ and reverse 5′-GAACGCAATCCGGAGTTTTA-3′. Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain size and purity. The PCR reactions using the LightCycler (Roche) apparatus were carried out in a final volume of 20 μl of a reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR Green I mix, 3 mM $MgCl_2$, and 1 μM of the primers, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and subjected to an initial denaturation at 95°C for 10 min, followed by 45 rounds of amplification at 95°C (10 s) for denaturation, 62°C (10 s) for annealing, and 72°C (10 s) for extension, with a temperature slope of 20°C/s. The transcript amount for the *ZIC2* gene was estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5′-CATCTCTGCCCCCTCTGCTGA-3′ and reverse 5′-GGATGACCTTGCCCACAGCCT-3′) transcript amount determined in corresponding samples.

Protein extraction

The cells were washed twice with cold phosphate-buffered saline, and were centrifuged briefly. The cell pellets were incubated at 4°C for 30 min in a lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS and 10 mM Tris pH 8.0) with

proteinase inhibitor cocktail (Roche Mannheim, Germany). The protein concentration was measured with BCA (Thermo, Rockford, IL, USA).

Western blot analysis

Samples (50 μg) of proteins were separated by SDS/PAGE in 4–12% gel and transferred to nitrocellulose membranes for reaction with antibodies against ZIC2 and β-actin. Secondary antibodies, horseradish peroxidase-conjugated rabbit anti-goat and rabbit anti-mouse IgG, were detected by using SuperSignal Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA).

IHC

IHC of 4-μm sections of paraffin-embedded specimens was performed using goat anti-ZIC2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, after deparaffinization and hydration, The endogeneous peroxidase activity was quenched by 30 min incubation in a mixture of 0.3% hydrogen peroxide solution in 100% methanol, after which the sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz) in phosphate buffered saline (PBS) before reacting with anti-ZIC2 antibody (1:100 dilution) at 4°C in a moist chamber overnight. Upon incubation with the primary antibody, the specimens were washed three times in PBS and treated with Histofine Simplestain Max-PO (G) (Nichirei, Tokyo, Japan) followed by color development in 3,3′-diaminobenzidine tetrahydrochloride (DAKO, Carpinteria, CA, USA). Finally, the slides were lightly counterstained with hematoxylin, dehydrated with ethanol, cleaned with xylene, and mounted. Non-specific binding of an antibody to proteins other than the antigen sometimes occurred. To avoid non-specific binding, an immunizing peptide blocking experiment was performed. As a negative control, triplicate sections were immunostained without exposure to primary antibodies, which confirmed the staining specificity. To quantify the state of ZIC2 protein expression in those components, we used IHC score systems described previously (Endo et al. 2004; Shimada et al. 2005; Saito et al. 2006; Onda et al. 2006; Kouzu et al. 2006; Kato et al. 2007; Nomura et al. 2009; Sakuma et al. 2006; Tanaka et al. 2003; Yamano et al. 2008). Briefly, the stained cells were determined in at least five random fields at 400× magnification in each section. The intensity of the ZIC2 immunoreaction in the cell was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. The cell number and the staining intensity then were multiplied to produce a ZIC2 IHC score. Cases with a score exceeding 64.6 (the highest score for normal tissue) were defined as ZIC2-positive. These judgments were made by two independent pathologists, neither of whom had any knowledge or information of the patients' clinical status.

Statistical analysis

The statistical significance of the ZIC2 expression levels was evaluated using the Mann–Whitney U test, with $P < 0.05$ considered statistically significant. The data are expressed as the mean ± SE. Disease-free survival time was defined as the time between tumor treatment and detection of the first locoregional recurrence, distant metastasis, or both, or the date of the last follow-up, whichever occurred first. The overall survival time was defined as the interval between the date of treatment and death or until the last objective follow-up information was obtained. Patients without evidence of disease (local recurrence or metastasis) during follow-up were considered to have a good prognosis; patients with local recurrence or distant metastasis during follow-up were considered to have a poor prognosis. Survival curves were obtained using the Kaplan–Meier method and differences in survival rates between ZIC2-positive and ZIC2-negative cases were compared by log-rank tests with 95% significance.

Results

Evaluation of *ZIC2* mRNA expression in OSCC-derived cell lines

We measured *ZIC2* mRNA expression in five OSCC-derived cell lines (HSC-2, HSC-3, OK92, H1, and Sa3) and HNOKs using qRT-PCR analysis. *ZIC2* mRNA was up-regulated significantly in all OSCC cell lines examined compared with the HNOKs (Fig. 1; $*P < 0.05$). These data were consistent with previous microarray data (Yamano et al. 2008).

Evaluation of ZIC2 protein expression in OSCC-derived cell lines

To investigate ZIC2 protein expression status in the OSCC-derived cell lines and the HNOKs, we performed Western blot analysis. Figure 1b shows representative results of the Western blot analysis. The molecular weight of the ZIC2 was 70 kDa. A significant increase of ZIC2 expression was observed in all OSCC cell lines compared with the HNOKs used as controls.

Evaluation of ZIC2 expression in primary OSCCs

We measured *ZIC2* mRNA expression in primary OSCCs and paired normal oral tissues from 74 patients. Similar to

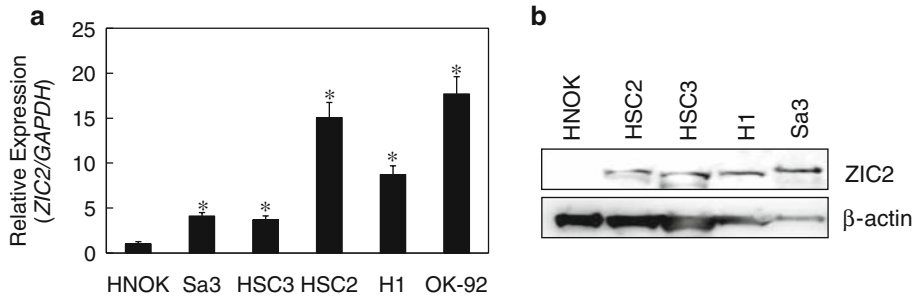

Fig. 1 **a** Quantification of ZIC2 mRNA and protein levels in OSCC-derived cell lines by qRT-PCR and Western blot analyses. Significant up-regulation of *ZIC2* mRNA is seen in five OSCC-derived cell lines compared with that in the HNOKs. Data are expressed as means ± SEM of values from three assays (* $P < 0.05$; Mann–Whitney's *U* test). **b** Western blot analysis of ZIC2 protein in the OSCC-derived cell lines and HNOKs. ZIC2 protein expression is up-regulated in OSCC-derived cell lines compared with HNOKs

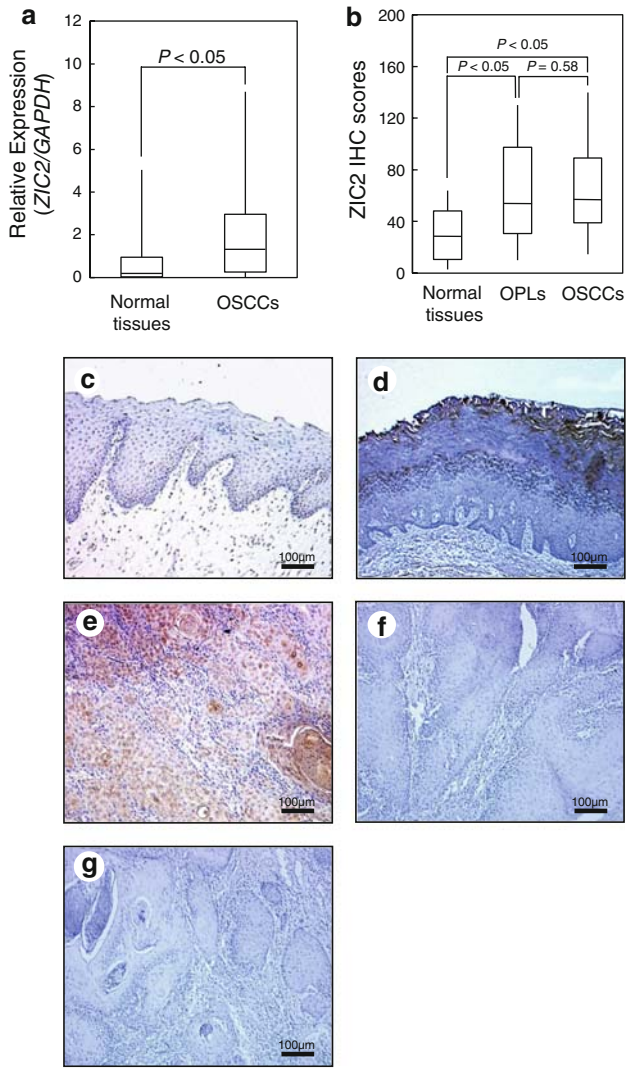

Fig. 2 ZIC2 expression status in primary OSCCs and representative IHC staining of ZIC2 in normal oral tissue, OPLs and primary OSCC. **a** Comparison of *ZIC2* mRNA expression levels between primary OSCCs and matched normal oral tissues. The relative mRNA expression levels in primary OSCCs ($n = 74$) and the matched oral tissues ($n = 74$) range from 0.01 to 8.69 (median 1.09) and 0.006 to 5.04 (median 0.172), respectively. Significantly higher *ZIC2* expression is found in primary OSCCs than matched normal oral tissues ($P < 0.05$; Mann–Whitney's *U* test). Data are expressed as the mean values ± SEM of two independent experiments with samples in triplicate. **b** The state of ZIC2 protein expression in normal oral tissues ($n = 74$), OPLs ($n = 20$) and primary OSCCs ($n = 74$). The ZIC2 IHC scores are calculated as follows: IHC score = 1 × (number of weak stained cells in the field) + 2 × (number of moderately stained cells in the field) + 3 × (number of intense stained cells in the field). The ZIC2 IHC scores for OSCCs, OPLs and normal oral tissues range from 14.63 to 140.1 (median 64.48), from 10.29 to 130.9 (median 55.01) and from 3.07 to 64.55 (median 30.12), respectively. ZIC2 protein expression levels in OSCCs and OPLs are significantly higher than that in normal oral tissues ($P < 0.05$; Mann–Whitney's *U* test). **c–e** Representative IHC results of ZIC2 in normal oral tissue, OPLs, and primary OSCC. **c** Normal oral tissue exhibits negative ZIC2 protein expression. Original magnification, ×100. **d** A ZIC2-positive case of OPL. The immunoreaction is enhanced in the spinous layer. Original magnification, ×100. **e** A ZIC2-positive case of OSCC. Positive immunoreaction for ZIC2 is detected in the nucleus and cytoplasm. Original magnification, ×100. Negative control staining by ZIC2 peptide blocking primary antibody (**f**) and without primary antibody (**g**). Original magnification ×100

the data from the OSCC-derived cell lines, qRT-PCR analysis showed that *ZIC2* mRNA expression was up-regulated in 49 (66%) of 74 primary OSCCs compared with the matched normal oral tissues. The relative mRNA expression levels in the normal oral tissues and primary OSCCs ranged from 0.006 to 5.04 (median 0.172) and 0.01–8.69 (median 1.09), respectively (Fig. 2a, $P < 0.05$).

In addition to mRNA expression in the primary OSCCs, we measured ZIC2 protein expression by IHC. Representative results for ZIC2 protein expression in normal oral tissue, OPLs and primary OSCC were shown in Fig. 2c–e, respectively. Strong ZIC2 immunoreactions were detected in OSCCs and OPLs, whereas normal oral tissues showed negative immunostaining. The ZIC2 IHC scores for OSCCs and normal oral tissues ranged from 14.63 to 140.1 (median 64.48) and 3.07 to 64.55 (median 30.12), respectively. The ZIC2 IHC scores in primary OSCC cells were significantly higher than those of normal tissues (Fig. 2b, $P < 0.05$). In

addition, ZIC2 IHC scores in OPLs ranged from 10.29 to 130.9 (median 55.01) and were also higher than those of normal oral tissues (Fig. 2b, $P < 0.05$). The correlation between the clinicopathologic characteristics of the patients with OSCC and the status of ZIC2 protein expression using the IHC scoring were shown in Table 1. Among the clinical classifications, ZIC2-positive OSCCs were correlated with the histopathologic types of OSCC (well differentiated, moderately differentiated, and poorly differentiated) (Table 1) ($P < 0.032$).

Effect of ZIC2 expression on patient survival

Survival analysis using the Kaplan–Meier method showed that ZIC2 up-regulation was a significant factor in disease-free survival (Fig. 3a; log-rank test, $P = 0.038$) and overall survival (Fig. 3b; log-rank test, $P = 0.034$). The disease-free survival rates in ZIC2 up-regulated cases and down-regulated cases were 66.71 and 87.84%, respectively. The overall survival rates in cases with up-regulated and down-regulated ZIC2 were 57.31 and 85.51%, respectively.

Table 1 Correlation between ZIC2 expression and clinical classification in OSCCs

Clinical classification	Total	Results of immunostaining		*P* value
		No. of patients		
		ZIC2(−)	ZIC2(+)	
Age at surgery				
<60	20	7 (35%)	13 (65%)	0.40
60–70	24	12 (50%)	12 (50%)	
70>	30	16 (53%)	14 (47%)	
Gender				
Male	51	27 (53%)	24 (47%)	0.21
Female	23	8 (35%)	15 (65%)	
T-primary tumor				
T1	9	3 (33%)	6 (67%)	0.69
T2	30	15 (50%)	15 (50%)	
T3	14	7 (50%)	7 (50%)	
T4	21	10 (48%)	11 (52%)	
N-regional lymph node				
N+	41	18 (54%)	19 (46%)	0.20
N–	33	17 (39%)	20 (61%)	
Stage				
I	7	2 (29%)	5 (71%)	0.64
II	16	8 (50%)	8 (50%)	
III	8	5 (63%)	3 (38%)	
IV	43	20 (47%)	23 (53%)	
Histopathological type				
Well differentiated	50	18 (37%)	32 (63%)	0.032*
Moderately differentiated	20	13 (60%)	7 (40%)	
Poorly differentiated	4	3 (75%)	1 (25%)	
Tumor site				
Gingiva	20	9 (45%)	11 (55%)	0.807
Tongue	38	19 (50%)	19 (50%)	
Buccal mucosa	7	3 (43%)	4 (57%)	
Oral floor	6	3 (50%)	3 (50%)	
Oropharyngeal isthmus	1	0 (0%)	1 (100%)	
Soft palate	1	1 (100%)	0 (0%)	

ZIC2(+) up-regulated ZIC2, *ZIC2(−)* down-regulated ZIC2

* $P < 0.05$

Discussion

We previously reported the gene expression profiling of OSCC to identify genes associated with oral carcinogenesis (Yamano et al. 2008). Using microarray analysis, *ZIC2* was one of the most significantly up-regulated genes in OSCC-derived cell lines. In this present study, we investigated the relationship between ZIC2 expression and clinical behaviors of patients with OSCC.

ZIC2 is widely known to be a transcriptional regulator (Brown and Brown 2009), and controlled dopamine receptor D1 is the most abundant dopamine receptor in the central nervous system (Yang et al. 2000), which regulates neuronal growth, development, and dopamine receptor D2-mediated events (Missale et al. 1998). Recent studies have reported the relationship between ZIC2 expression and malignant tumors, such as lung cancer, endometrial cancer, pediatric medulloblastoma and synovial sarcoma (Gure et al. 2000; Bidus et al. 2006; Pfister et al. 2007; Fernebro et al. 2006). However, detail mechanisms of ZIC2 in those cancers were unclear. Therefore, studies of ZIC2 function in OSCCs are important.

In this study, significant up-regulation of *ZIC2* mRNA was observed in OSCCs and in five OSCC-derived cell lines, compared with the matched normal counterparts. These results were consistent with previous report of endometrial cancer (Bidus et al. 2006). By Western blot and IHC, in addition to *ZIC2* mRNA expression, ZIC2 protein level was significantly up-regulated in primary OSCCs and OSCC-derived cell lines compared with matched normal counterparts. Up-regulation of ZIC2 was also detected even in the OPLs, suggesting that dysregulation of protein expression was seen in the premalignant stage. Futhermore, this study showed that high levels of ZIC2 expression were associated significantly with lower disease-free survival (Fig. 3a) and overall survival (Fig. 3b) rates. Therefore, these results indicated that ZIC2 might have the function as a prognosis indicator for OSCC. In contrast, ZIC2 protein expression was correlated with the degree of differentiation

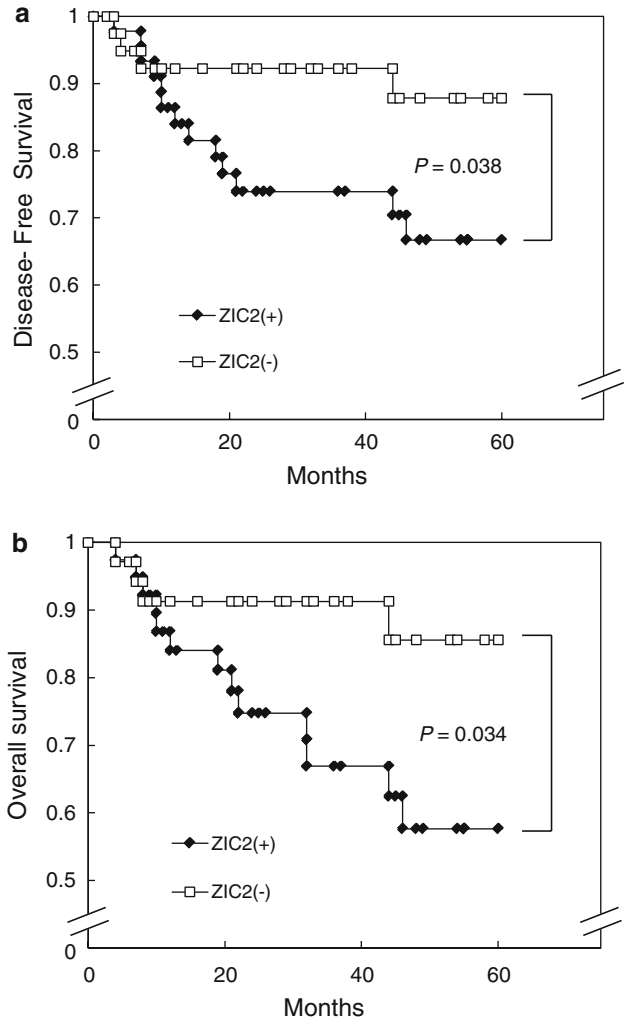

Fig. 3 Kaplan–Meier survival curve for disease-free survival and overall survival rate of patients with OSCC. High levels of ZIC2 expression are significantly associated with **a** lower disease-free survival and **b** overall survival rates (**a** $P = 0.038$; **b** $P = 0.034$, respectively). The log-rank statistic is used to test the difference in survival times between the groups. *ZIC2(+)* up-regulated ZIC2, *ZIC2(−)* down-regulated ZIC2

in OSCCs. Generally, prognosis of well-differentiated cancers is better than that of poor-differentiated cancers (Nishida et al. 1999). However, prognosis is associated with not only differentiation type but also tumor size and status of metastasis. Therefore, further studies with more clinical samples are needed to address in greater detail the status of ZIC2 in oral carcinogenesis and prognosis.

In conclusion, our results indicated that ZIC2 is frequently overexpressed not only in OSCCs but also in OPLs. In addition, up-regulated ZIC2 is associated with 5-year survival rate. Thus, we suggest here that ZIC2 might play an important role in the course of oral tumorigenesis and the prognosis of OSCC.

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Conflict of interest statement We declare that we have no conflict of interest.

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