Induced Pluripotent Stem Cell-related Genes Correlate With Poor Prognoses of Oral Squamous Cell Carcinoma

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Abstract. *Background/Aim:* We recently investigated the contribution of the iPS-related genes *SOX2*, *OCT4*, and *Nanog* to de-differentiation by assaying for their mRNA levels. Given that mRNA expression does not always correlate with the protein levels, the aim of this study was to retrospectively determine the expression of these four iPS-related factors in human OSCC specimens by immunohistochemistry and examine their association with patient prognosis. *Materials and Methods:* iPS cell-related gene expression in 89 OSCC patients by tissue microarray, and its correlation with clinicopathological factors, differentiation, metastasis, and poor prognoses were investigated. *Results:* No evidence of statistically significant relationships was found between the expression of iPS cell-related genes and clinicopathological parameters. However, our data indicated that *KLF4* expression was associated with survival, and poor tumor differentiation. In addition, high expression of *KLF4* was an independent poor prognostic factor ($p=0.004$) for OSCC patients. *Conclusion:* In preoperative biopsies, higher *KLF4* and poor differentiation may be clinically effective predictors for the prognosis of oral cancer.

Oral squamous cell carcinoma (OSCC) is a malignancy characterized by genomic instability and dysregulated cell growth attributable to oncogene overexpression, repression of tumor-suppressor genes, and other genetic, epigenetic, and microRNA alterations (1). These changes are associated with poor prognosis in patients with OSCC,

especially when locoregional recurrence or metastasis to regional lymph nodes or distant sites are present (2). However, the mechanisms of metastasis and recurrence in OSCC remain unclear.

Stem cells have the ability to self-renew and generate mature cells with different fates in tissues and are currently in focus for their role in cancer biology. Cancer stem cells (CSCs) share some of the fundamental features of normal stem cells, such as self-renewal and differentiation capacity, and are thus thought to contribute to tumor recurrence and resistance to therapy (3-6).

Induced pluripotent stem (iPS) cells have been shown to acquire pluripotency following the introduction of four transcriptional factors: octamer-binding transcription factor 4 (*OCT4*), sex determining region Y-box 2 (*SOX2*), avian myelocytomatosis viral oncogene (*C-myc*), and Kruppel-like factor 4 (*KLF4*), which can also induce pluripotency in embryonic or adult murine fibroblasts (7, 8). These pluripotency-inducing factors have been shown to play an important role in embryonic stem cells, normal stem cells, and CSCs, including in OSCC (9). While necessary for the acquisition of pluripotency, it has also been suggested that these factors may have an oncogenic potential (10).

Although the status and functional roles of these genes in CSCs have not been elucidated for all malignancies, their expression is associated with several types of cancers, including esophageal, colon, lung, and bladder cancers (11-14).

We recently investigated the contribution of the iPS-related genes *SOX2*, *OCT4*, and *Nanog* to de-differentiation by assaying for their mRNA level (5). *OCT4* and *SOX2* mRNA is expressed in esophageal squamous carcinoma cell lines (15), while *KLF4* and *C-myc* mRNA is elevated in dysplastic epithelium and laryngeal and oral SCC (16).

Given that mRNA expression does not always correlate with the protein levels, we retrospectively determined the expression of these four iPS-related factors in human OSCC specimens by immunohistochemistry and examined its association with patient prognosis.

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Key Words: Squamous cell carcinoma, induced pluripotent stem cells, reprogramming factors, iPS cell-related factors, kruppel-like factor 4.

Materials and Methods

Patients and clinical specimens. Eighty-nine patients with OSCC were retrospectively included in this study. All underwent surgical resection at the Department of Oral and Maxillofacial Surgery, Kobe University Hospital between July 2011 and December 2013 and were followed up until August 2017. Clinical and pathological data were retrospectively collected from the hospital records. All participants received clinical treatment according to consensus guidelines for head and neck cancers [UICC classification, 7th edition (2009)]. Each patient underwent surgical excision of tumors with a clear 10-mm margin from the tumor edge. All tumor specimens were independently processed by two experienced pathologists. The protocol for this study was approved by the Ethics Committee of Kobe University. Because of the retrospective nature of this study, informed consent was not required. Instead, patients were given the opportunity to refuse use of their samples in this study.

Histopathology and immunohistochemistry. Formalin-fixed paraffin-embedded tumor sections were prepared at the department of pathology of our hospital and cut into 4- μ m sections. The sections were subsequently deparaffinized in xylene and rehydrated in a graded alcohol series, washed three times for 3 min in phosphate buffered saline (PBS), and subjected to antigen retrieval by proteinase k treatment (Dako, Carpinteria, CA, USA) for 15 min prior to blocking of endogenous peroxidase activity by incubation with 3% H₂O₂ for 10 min at room temperature. Following two 5-min washes in PBS, the sections were incubated overnight at 4°C with the following primary antibodies in Can Get Signal Immuno-stain Solution A (Toyobo, Osaka, Japan): polyclonal rabbit anti-mouse/human OCT4 (#09-0023; STEMAGENT, Cambridge, MA, USA), polyclonal rabbit anti-mouse/human SOX2 (#09-0024; STEMAGENT), monoclonal rabbit anti-mouse/rat/human C-myc (#ab32072; Abcam, Cambridge, UK), and monoclonal mouse anti-mouse/human KLF4 (#09-0021; STEMAGENT). The antibodies were used at the following dilutions: SOX2, 1:100; KLF4, 1:100; OCT4, 1:100; and C-myc, 1:100. Following three 3-min washes in PBS after primary antibody treatment, sections were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG polyclonal antibody (#424142; Nichirei Bioscience, Tokyo, Japan) or anti-mouse IgG polyclonal antibody (#424132; Nichirei Bioscience) for 120 min at room temperature without dilution. Following three 3-min washes in PBS, the signal was developed as a brown reaction product using the peroxidase substrate 3,3'-diaminobenzidine (#415171; Nichirei Bioscience) for 5 min at room temperature. Following two 3-min washes in distilled water, sections were counterstained with hematoxylin for 4 min and images were captured using a BZ-X700 confocal microscope (Keyence Corporation, Osaka, Japan). Cancerous tissues and normal epithelium were imaged by light microscopy at $\times$ 200 magnification for each patient. The expression of each factor was determined independently, and cancerous and normal tissues were scored using BZ-H3C/Hybrid cell count (Keyence, Osaka, Japan). Finally, the percentages of stained cells in cancerous versus normal tissues was calculated.

Sets of normal and cancerous tissues from the same patient were selected and stained simultaneously to standardize the controls, for the analysis of expression of each of the four genes. The discriminatory ability of iPS-related gene intensity (high vs. low expression) as an

Table I. Percentages of positively-stained cells in cancerous versus normal tissues.

Factor	Positive stained nuclei, %		
	Low	Cutoff values ^a	High
SOX2	$\leq$	100.5	<
KLF4	$\leq$	118.1	<
OCT4	$\leq$	102.4	<
C-myc	$\leq$	106.7	<

^aCutoff values were determined by a receiving operating characteristic curve.

indicator of disease-specific survival (DSS) was evaluated using a receiving operating characteristic (ROC) curve to determine the cutoff values for clinical tests. The area under the curve (AUC) was used to measure the accuracy of this discrimination and ranged from 0.5 to 1. Cutoff values were selected to minimize the number of false positive and false negative results. The expression levels of the iPS-related factors were used to determine the cutoff values for low and high expression using the ROC curve method described above. The cutoff values for SOX2, KLF4, OCT4, and C-myc were 100.5%, 118.1%, 102.4%, and 106.7%, respectively (Table I). Representative immunohistochemistry results are shown in Figure 1.

Statistical analysis. SPSS 22.0 (IBM, Armonk, NY, USA) and Ekuseru-Toukei 2012 (Social Survey Research Information Co., Ltd., Tokyo, Japan) software were used for all statistical analyses. Associations of each variable were analyzed by Fisher's exact test or the chi-square test for categorical variables. Associations between preoperative variables, including iPS-related genes (SOX2, KLF4, OCT4, C-myc), with DSS were introduced into a multiple logistic regression model. Forward stepwise algorithms were used to reject variables that did not significantly fit the model. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated, and overall survival (OS), DSS, distant metastasis (DM), and local control (LC) rates following surgery were estimated for each group using the Kaplan-Meier method, with differences assessed using the log-rank test. For all analyses, a value of $p < 0.05$ was considered to indicate statistical significance.

Results

Patient characteristics. Clinical characteristics of enrolled patients with OSCC are summarized in Table II. Among the 89 patients, 51 were male (57.3%) and 38 were female (42.7%), and the mean age was 66.7 $\pm$ 14.2 years (range=22-88 years). Clinical T classifications were T1 in 28 patients (31.5%), T2 in 36 patients (40.4%), T3 in 9 patients (10.1%), T4a in 13 patients (14.6%), and T4b in 3 patients (3.4%). The most common primary tumor site was the tongue (46 patients/51.7%). The 3-year disease control status was local recurrence in 12 patients (13.5%), regional recurrence in 20 patients (22.5%), DM in 9 patients (10.1%), and disease-specific death in 8 patients (9.0%) (Table II).

Figure 1. Expression levels of iPS-related genes SOX2, KLF4, OCT4, and C-myc by immunohistochemistry. Images show representative examples of low expression (A–D), high expression (E–H), and normal epithelium (I–L); magnification: $\times 200$. Scale bar, 50 μm .

Expression of iPS-related genes. iPS-related genes are predominantly localized to the nucleus of OSCC cells, and immunostaining of the four proteins examined in our study

(SOX2, KLF4, OCT4, and C-myc) showed that they were primarily expressed in nuclei. However, in some instances, the nuclei and cytoplasm were stained to the same degree

with antibodies against SOX2, KLF4, and OCT4 in OSCC cells. All four proteins were also detected in normal basal epithelial cells (Figure 1).

Association between the expression of iPS-related genes and clinicopathological factors. Next, the associations between these transcription factors and clinicopathological factors were investigated (Table III). KLF4 and C-myc expression were significantly associated with age ($p=0.019$ and $p<0.001$, respectively) and exposure to tobacco ($p=0.016$ and $p=0.036$, respectively), while SOX2 and OCT4 showed no statistically significant associations with these factors. OCT4 expression was significantly higher in patients with multiple neck metastases than in patients without multiple neck metastases ($p=0.020$). Additionally, OCT4 expression was significantly higher in patients with moderate or poor differentiation than in those with well-differentiated tumors ($p=0.044$). There were no statistically significant relationships between the iPS-related factors and other clinicopathological parameters, including alcohol, clinical T stage, clinical N stage, PS, primary tumor site, or extra nodal extension (ENE).

Correlation between iPS-related genes and survival rates. The 3-year OS rates of patients with high KLF4 and OCT4 expression were significantly decreased compared to those with low expression ($p=0.002$ and $p=0.015$, respectively) (Figures 2-3). The 3-year DSS rates of patients with high KLF4, OCT4, and SOX2 expression were significantly decreased compared with those exhibiting low expression $p<0.001$, $p=0.010$, and $p=0.007$, respectively) (Figures 2-4). Regarding 3-year LC rates, high KLF4 expression was associated with a decreased rate compared with low expression ($p=0.012$) (Figure 2). Finally, the 3-year DM rates of patients with high KLF4, OCT4, SOX2, and C-myc expression were decreased compared to those exhibiting low expression ($p<0.001$, $p=0.030$, $p=0.004$, and $p<0.001$, respectively) (Figures 2-5). Multivariate analysis also revealed that KLF4 (OR=10.28) and tumor differentiation (OR=14.85) were the most predictive factors for DSS ($p=0.004$) (Table IV).

Discussion

In recent years, stem cells have been identified in most tissue types, and CSCs have been hypothesized to enable metastasis. Research to identify the genetic changes leading to cancer has led to major advances in CSC biology (3). iPS technology (the mechanism of action of iPS-related factors) has been demonstrated in mouse fibroblasts by reprogramming with four transcription factors, SOX2, KLF4, OCT4, and C-myc, with Nanog now reported to be less relevant (7), and the possibility of these iPS-related genes being associated with malignant transformation has been proposed (10). For example, KLF4 has been associated with both tumor

Table II. Clinicopathological characteristics of the 89 oral squamous cell carcinoma patients.

Characteristics of patients	No. of patients	(%)
Age, years		
<65	34	38.2
≥65	55	61.8
Gender		
Male	51	57.3
Female	38	42.7
Exposure to tobacco		
Smoker	27	30.3
Non	62	69.7
Exposure to alcohol		
Drinker	39	43.8
Non	50	56.2
PS		
≤1	82	92.1
>2	7	7.9
Primary tumor site		
Upper gingiva	9	10.1
Lower gingiva	16	18.0
Buccal mucosa	6	6.7
Tongue	46	51.7
Oral floor	12	13.5
Palate	0	0
Clinical T stage		
I	28	31.5
II	36	40.4
III	9	10.1
IVA/B	16	18.0
Clinical N stage		
0 or I	67	75.3
II or III	22	24.7
ENE		
Positive	13	14.6
Negative	26	29.2
Non-lymph node metastasis, Non-neck dissection	50	56.2
Multiple neck metastasis		
Positive	18	20.2
Negative	37	41.6
Non-neck dissection	34	38.2
Margin		
Positive	25	28.1
Negative	64	71.9
Tumor differentiation		
Well	57	64.1
Moderate	27	30.3
Poor	4	4.5
Others	1	1.1
Disease control status		
Survival	71	79.8
Local recurrence	12	13.5
Regional recurrence	20	22.5
Distant metastasis	9	10.1
Disease specific death	8	9.0

ENE: Extra nodal extension.

Table III. Clinicopathological characteristics of the 89 patients according to the expression of each gene.

	SOX2 expression				KLF4 expression			OCT4 expression			C-myc expression		
	n	Low (n; %)	High (n; %)	p-Value	Low (n; %)	High (n; %)	p-Value	Low (n; %)	High (n; %)	p-Value	Low (n; %)	High (n; %)	p-Value
Age,years				0.051			0.019*			0.193			<0.001*
<65	34	11	23		21	13		16	18		10	24	
≥65	55	30	25		47	8		34	21		39	16	
Gender				0.390			0.076			0.390			0.397
Male	51	21	30		35	16		28	23		26	25	
Female	38	20	18		33	5		22	16		23	15	
Exposure to tobacco				0.165			0.016*			0.646			0.036*
Smoker	27	9	18		16	11		14	13		10	17	
Non	62	32	30		52	10		36	26		39	23	
Exposure to alcohol				1.000			1.000			0.397			0.391
Drinker	39	18	21		30	9		24	15		19	20	
Non	50	23	27		38	12		26	24		30	20	
PS				0.241			0.666			0.461			1.000
0 or 1	82	36	46		63	19		45	37		45	37	
More than 2	7	5	2		5	2		5	2		4	3	
Primary tumor site				0.673			0.139			0.831			0.671
Tongue	46	20	26		32	14		25	21		24	22	
Otherwise	43	21	22		36	7		25	18		25	18	
Clinical T stage				1.000			0.407			1.000			0.639
T1,T2	64	29	35		47	17		36	28		34	30	
T3,T4	25	12	13		21	4		14	11		15	10	
Clinical N stage				1.000			1.000			0.808			0.806
0 or 1	67	31	36		51	16		37	30		36	31	
>2	22	10	12		17	5		13	9		13	9	
ENE				0.495			0.157			0.734			0.307
Positive	13	4	9		6	7		5	8		4	9	
Negative	26	12	14		19	7		13	13		14	12	
Multiple neck metastasis				0.774			0.115			0.020*			0.391
Positive	18	7	11		10	8		5	13		7	11	
Negative	37	17	20		29	8		24	13		20	17	
Tumor differentiation				0.271			0.801			0.044*			0.125
Well	57	29	28		44	13		37	20		35	22	
Moderate, Poor, Others	32	12	20		24	8		13	19		14	18	

*Statistically significant ($p<0.05$). ENE: Extra nodal extension.

suppression and oncogenesis (17, 18, 19), with increased expression reported in human head and neck squamous cell carcinoma (HNSCC) and breast cancer, and KLF4 has been demonstrated to be a poor prognostic factor in skin cancer and early breast cancer (20). SOX2 and OCT4 are important for the enhancement of self-renewal and pluripotency in pluripotent stem cells (21, 22). C-myc is an oncogenic transcription factor involved in the regulation of cell proliferation, differentiation, and apoptosis, and it also plays an important regulatory role in the activity of genes involved in cell division (16, 23).

Therefore, the expression of iPS-inducing factors in human OSCC specimens was investigated by immunohistochemistry, and the associations between these factors and patient prognosis were analyzed.

Table IV. Predictive factors for disease-specific survival in oral squamous cell carcinoma patients by multivariate analysis.

Disease specific survival	Odds ratio	95%CI		p-Value
		Lower	Upper	
KLF4	10.28	2.069	51.098	0.004*
Tumor differentiation	14.85	1.822	120.943	0.012*

*Statistically significant ($p<0.05$). CI: Confidence interval.

A previous study showed that there was no statistically significant association between KLF4 and C-myc expression when stratified by age in patients with esophageal, gastric,

Figure 2. Relationships between (a) OS, (b) DSS, (c) LC, and (d) DM rates of patients with oral squamous cell carcinoma and KLF4 expression level (high or low). *Statistically significant ($p < 0.05$).

or lung cancer (24-26). Additionally, a relationship between increased age and KLF4 expression in breast cancer patients has been identified (27). In patients with OSCC, no differences in KLF4 or C-myc expression were found when stratified by age or exposure to tobacco (9, 20, 28). In the present study, KLF4 and C-myc expression were significantly associated with age <65 years, a finding inconsistent with previous reports. To the best of our knowledge, difference in KLF4 expression levels by age group has not been previously reported. A previous study showed that C-myc expression was significantly different

between patients with and without tobacco exposure (1). The present study also showed an association between C-myc and tobacco.

SOX2 and OCT4 have been significantly associated with poor tumor differentiation in esophageal and breast cancers (4, 15, 29), while in oral cancer, SOX2 and OCT4 have been positively associated with lymph node metastasis and poorer tumor differentiation (30-32). In the present study, only OCT4 expression was significantly associated with multiple neck metastases and poor tumor differentiation, consistent with its reported association with lymph node metastasis and

Figure 3. Relationships between (a) OS, (b) DSS, (c) LC, and (d) DM rates of patients with oral squamous cell carcinoma and OCT4 expression level (high or low). *Statistically significant ($p < 0.05$).

tumor stage. Together, these data suggest that OCT4 may be involved in tumor metastasis.

Studies have shown that SOX2 and OCT4 are important transcription factors involved in maintaining self-renewal and pluripotency in CSCs; high SOX2 and OCT4 expression might also be associated with carcinogenesis in various cancers (11, 33, 34). High OCT4 expression has been associated with poor disease-free survival and OS in esophageal and cervical cancer (11, 34). Additionally, OCT4 may contribute to unfavorable prognosis in malignancies of the stomach, esophagus, lungs, and oral cavity by increasing

tumor invasion and metastasis and by decreasing tumor differentiation (11, 31-32, 35-36). SOX2 has previously been associated with DM and DSS in oral cancer (9, 32), and OCT4 expression has been associated with poor tumor differentiation, tumor invasion, and metastasis (30-31). Conversely, high SOX2 expression has been shown to be associated with increased survival rates (37). Our findings indicate that high SOX2 and OCT4 expression are significantly associated with decreased 3-year DSS and DM rates of OSCC patients compared with those having low expression of these factors, consistent with the previous studies described above.

Figure 4. Relationships between (a) OS, (b) DSS, (c) LC, and (d) DSS rates of patients with oral squamous cell carcinoma and SOX2 expression level (high or low). *Statistically significant ($p<0.05$).

C-myc is a member of the Myc family of proto-oncogenes, that also includes L-myc and N-myc (23). The C-myc protein is a transcription factor, aberrant expression of which is sufficient to induce cell cycle progression. Moreover, C-myc is associated with poor tumor prognosis and CSC self-renewal (1). In esophageal squamous cell carcinoma, C-myc expression was significantly correlated with invasion depth, lymph node metastasis, and poor prognosis (38), while in oral cancer, C-myc overexpression was a clear indicator of poor prognosis and stage in locally advanced tumors (39). However, C-myc did not correlate with 5-year survival in patients with oral and oropharyngeal

carcinomas (40). Similarly, our results did not show any significant association between C-myc expression and 3-year DSS, although there was a tendency toward poorer prognoses in the high-C-myc expression group.

KLF4 is a bi-functional transcription factor that has opposing roles in different cancers, either as a tumor suppressor or oncogene. Increased KLF4 expression has been associated with poor prognoses in early-stage breast cancer, skin cancer, and HNSCC; furthermore, persistent KLF4 expression induced multi-drug resistance and was associated with progression and poor prognosis in HNSCC (20). Additionally, high KLF4 staining has been

Figure 5. Relationships between (a) OS, (b) DSS, (c) LC, and (d) DSS rates of patients with oral squamous cell carcinoma and C-myc expression level (high or low). *Statistically significant ($p < 0.05$).

significantly associated with moderately and poorly differentiated tumors and metastasis in human skin cancer (41). KLF4 overexpression in the skin has also been shown to result in hyperplasia and dysplasia, eventually leading to squamous cell carcinoma (18). Furthermore, KLF4 has been associated with low 3-year DSS rates in patients with advanced HNSCC. Additionally, KLF4 has been associated with DM and disease-free survival rates. KLF4 also has a statistically significant association with neck cancer recurrence, and a multivariate analysis showed that KLF4 was an independent predictor for worse DSS (20). In the

present study, decreased 3-year OS, DSS, LC, and DM rates among OSCC patients were significantly associated with high KLF4 expression compared to low expression. Regarding DSS rates, our results were consistent with those of previous studies. Our multivariate analysis also revealed that KLF4 (OR=10.28) and tumor differentiation (OR=14.85) were the most predictive factors for DSS ($p < 0.01$). Therefore, in preoperative oral cancer biopsies, higher KLF4 and poor differentiation may represent clinically effective predictors of the prognosis of oral cancer.

Conversely, KLF4 has been shown to be expressed in the cytoplasm and nuclei of gastric cancer cells, and loss of KLF4 has been associated with a poor prognosis (42). KLF4 has been reported to be predominantly located in the nuclei of oral cancer cells, and decreased KLF4 has been associated with poor prognosis (28). Furthermore, several functional investigations have indicated that decreased KLF4 expression was associated with the initiation and formation of precancerous lesions in various tissues (17, 43-47). Increased KLF4 expression induced cell-cycle arrest and apoptosis in gastric cancer cells, and KLF4 has been shown to induce apoptosis in bladder cancer, colon cancer, and leukemia cells (48). However, the apoptotic mechanism induced by KLF4 has not yet been demonstrated. In summary, further investigations are necessary to clarify the association between KLF4 expression and prognosis.

In conclusion, the expression levels of four iPS-related genes (*SOX2*, *KLF4*, *OCT4*, and *C-myc*) were investigated in tumor samples from patients with OSCC, and the associations between their expression levels and patient outcomes were examined. Our findings indicate that high expression of these genes may be associated with DM in OSCC, and future postoperative pathological studies of iPS-related gene expression may therefore influence treatment strategy, specifically the control of distant metastasis. Furthermore, high KLF4 expression and tumor differentiation were found to be independent poor prognostic factors for OSCC. Therefore, in preoperative biopsies, higher KLF4 expression and poor differentiation may represent clinically effective predictors for the prognosis of oral cancer.

Conflicts of Interest

The Authors declare that they have no competing interests regarding this study.

Authors' Contributions

DT and TH conceived and designed the experiments; YM performed the experiments; EI, IS, and RA provided experimental assistance; YM, DT, and TH analyzed the data; TU contributed reagents, materials, and analysis tools; TU provided advice on experimental techniques; YM and TH wrote the manuscript; TK proofread the manuscript; AS, AM, and TK supervised all aspects of this study.

Acknowledgements

The Authors thank James P. Mahaffey, PhD, and Clare Cox, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript. This study was funded by JSPS KAKENHI (Grant Number 16K20575). The funders had no role in the design of the study, the collection, analysis and interpretation of data, or manuscript writing. The Ethics Committee of Kobe University Hospital approved this study (permission number: 180171), and written informed consent was obtained from all patients.

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Received January 8, 2019

Revised January 24, 2019

Accepted January 25, 2019