Expression Analysis of iPS Cell – Inductive Genes in Esophageal Squamous Cell Carcinoma by Tissue Microarray

YUTAKA SHIMADA[1], TOMOYUKI OKUMURA[1], SHINICHI SEKINE[1], MAKOTO MORIYAMA[1], SHIGEAKI SAWADA[1], KOSHI MATSUI[1], ISAKU YOSHIOKA[1], SHOZO HOJO[1], TORU YOSHIDA[1], TAKUYA NAGATA[1], JUNYA FUKUOKA[2] and KAZUHIRO TSUKADA[1]

Departments of [1]Surgery and Science, and [2]Surgical Pathology
Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Toyama, Japan

Abstract. *Aim: To understand the role of iPS inductive genes in esophageal cancer, we examined the expression of Sex determining region Y-box 2 (SOX2), Octamer-binding transcription factor 3/4 (OCT3/4), Krueppel-like factor 4 (KLF4), c-Myelocytomatosis viral oncogene (c-MYC) and Tir Na Nog (NANOG) using an esophageal squamous cell carcinoma tissue micrroarray. Materials and Methods: The immunohistochemical expression levels of the five genes were compared to the clinicopathological data of the 81 patients with esophageal cancer. Results: There was no relationship between the expression of the five genes and TNM factors of the patients. High expression of NANOG was an independent favorable prognostic factor (p=0.041). Among the patients who received postoperative cisplatin-based chemotherapy, patients with NANOG-positive tumor had significantly better prognosis than those whose tumors were NANOG negative (p=0.024). On the other hand, those with c-MYC-positive expression tended to have a worse prognosis and were resistant to cisplatin-based chemotherapy. Conclusion: NANOG expression was found to be an independent prognostic factor for patient with esophageal cancer. Patients with NANOG-positive expression tumor may be good candidates for cisplatin-based treatment.*

Induced pluripotent stem cells (iPS) are cells that have acquired pluripotency due to the introduction of genes such as Sex determining region Y-box 2 (*SOX2*), Octamer-binding transcription factor 3/4 (*OCT3/4*), Krueppel-like factor 4 (*KLF4*), c-Myelocytomatosis viral oncogenec (*c-MYC*) and Tir Na Nog (*NANOG*) and Lin-28 homolog (*LIN28*) (1, 2). Although these factors are necessary for the acquisition of pluripotency by stem cells, they have also been suggested to have oncogenic potential for normal cells (3). Aoi *et al.* reported that gastrointestinal cells had less potential for carcinogenesis than did fibroblasts (4). Thus, the role of iPS-inductive genes in normal epithelial cells, normal mesenchymal cells and cancer cells is unclear. Furthermore, the influence of these factors on the proliferation and metastatic potential of cancer is also unclear.

We previously suggested that *NANOG* stimulates the growth and metastasis of breast cancer cells, whereas *KLF4* inhibits these processes (5). In this study, we evaluated the expression of iPS-inducing factors in human esophageal squamous cell carcinoma (SCC) specimens by immunohistochemistry using a tissue mocroarray, and analyzed the association of these factors with the prognosis of the patients.

Materials and Methods

Tissue microarray. We used an esophageal SCC tissue microarray. A total of 114 patients with esophageal SCC from 1990 to 2008 were included. The tumor areas were selected with matched Hematoxylin and Eosin (HE) -stained slides and marked directly on the donor block. Each cylindrical tissue sample was cored (0.6 mm diameter) from the selected region in the donor block and extruded directly into the recipient block. Multiple 4 μm sections were cut with a microtome and transferred to glass slides (Super frost Plus, Fisher Scientific GmbH, Schwerte, Germany).

Patients. Out of 114 patients with esophageal SCC, 10 underwent R1 resection, two died in hospital, 13 underwent preoperative chemoradiation, and eight underwent preoperative chemotherapy; these patients were excluded from this study. Data of the remaining 81 patients with esophageal SCC were analyzed in this study. All eligible patients underwent R0 resections and did not die in hospital. The median follow-up time was 40 months. There were 72 male and

Abbreviations: Esophageal squamous cell carcinoma (ESCC), Induced pluripotent stem cells (iPS).

Correspondence to: Yutaka Shimada, MD, Ph.D., FACS, Department of Surgery & Science, Graduate School of Medicine & Pharmaceutical Sciences for Research, University of Toyama, Sugitani 2630, Toyama, 930-0194, Japan. Tel: +81 764347331, Fax: +81 764345043, e-mail: yshimada@med.u-toyama.ac.jp

Key Words: OCT 3/4, *KLF4*, *SOX2*, *c-MYC*, *NANOG*, esophageal cancer.

Table I. *Characteristics of the patients according to the expression of each gene.*

	KLF4			OCT3/4			SOX2			c-MYC			NANOG		
	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value
Years															
>65	28	12	0.166	28	12	0.941	28	12	0.617	19	21	1	21	17	1
<65	22	18		27	12		30	10		19	21		21	17	
Gender															
Male	45	26	0.648	47	23	0.182	50	21	0.243	33	38	0.608	37	30	0.985
Female	5	4		8	1		8	1		5	4		5	4	
T															
T1	8	10	0.14	11	6	0.283	13	5	0.698	9	9	0.321	11	5	0.529
T2	10	3		12	1		11	2		9	4		8	5	
T3	23	15		25	13		27	11		16	22		18	18	
T4	9	2		7	4		7	4		4	7		5	6	
N															
N0	15	12	0.36	19	7	0.64	20	7	0.822	15	12	0.303	13	11	0.896
N1	35	18		36	17		38	15		23	30		29	23	
M															
M0	45	27	1	50	21	0.644	53	19	0.504	35	37	0.551	38	31	0.916
M1	5	3		5	3		5	3		3	5		4	3	
TNM stage															
1	5	6	0.799	7	3	0.726	8	3	0.86	6	5	0.273	7	2	0.557
2a	9	5		11	3		11	3		9	5		6	7	
2b	7	4		9	2		9	2		7	4		7	4	
3	24	12		23	13		25	11		13	23		18	18	
4a	5	3		5	3		5	3		3	5		4	3	
Histology															
Well/mod	45	20	0.01	48	17	0.078	47	18	0.936	32	33	0.519	34	28	0.876
Poor	5	10		7	7		11	4		6	9		8	6	
Postoperative chemotherapy															
No	22	12	0.726	22	11	0.629	25	9	0.859	16	18	0.946	17	14	0.951
Yes	28	18		33	13		33	13		22	24		25	20	

KLF4: n=80; OCT3/4: n=79; SOX2; n=80; c-MYC: n=80; NONOG: n=76.

9 female patients. The average age of the patients was 64.5 years. TNM stage (ver 6) of the patients were as follows: stage 1: 11; stage 2a: 14; stage 2b: 12; stage 3: 36; and stage 4: 8. All M1 cases were cases with distant lymph node metastases only and these were removed by surgery. Forty-six patients underwent postoperative cisplatin-based chemotherapy. Of these, 40 patients received cisplatin plus 5-fluorouracil (FP) regimen and the other six patients received cisplatin alone or a FP plus doxorubicin hydrochloride regimen. The Institutional Review Board at the University of Toyama approved this study (#20-57).

Immunohistochemistry. Antibodies for the immunohistochemical staining were selected as follows. *c-MYC* (ab32; Abcam, Cambridge, UK), *OCT4* (ab19857; Abcam), *SOX2* (AB5603; Millipore, Billerica, MA, USA), *KLF4* (SC-20691; Santa Cruz, CA, USA), *NANOG* (IHC-00205; Bethyl Laboratories, Inc. Montogomery, TX, USA).

Glass slides with the primary antibodies were incubated at an optimized titer and diluted using Universal Blocking Reagent (BioGenex, Fremont, CA, USA) for 60 min. After washing three times with phosphate buffered saline (PBS), the slides were incubated for 30 min with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) diluted to 1:250 by Universal Blocking Reagent, washed three times in PBS, and then incubated for 45 min with Avidin–Biotin Complex Method Reagent (Vectastain Elite ABC Kit; Vector Laboratories). The reaction products were then rinsed twice with PBS, placed in 0.05 M Tris–HCl buffer (pH 7.5) for 5 min, and then developed with liquid 3,3-diaminobenzidine (Dako, Glostrup, Denmark) for 3 min. After the development, the slides were washed twice with distilled water, lightly counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted with resinous mounting medium. All procedures were carried out at room temperature (5).

Immunohistochemical analysis. Two researchers analyzed the expression of each gene independently, and scored the intensity of expression as 0 (no expression), 1 (weak expression), 2 (moderate expression), or 3 (strong expression). They also scored the distribution of expression as 0 (none), 1 (1-50% of tumor cells), or 2 (51-100% of tumor cells). Expressions of genes were evaluated by the sum total of the staining intensity and distribution. On the basis of the total score, each patient was then classified into one of

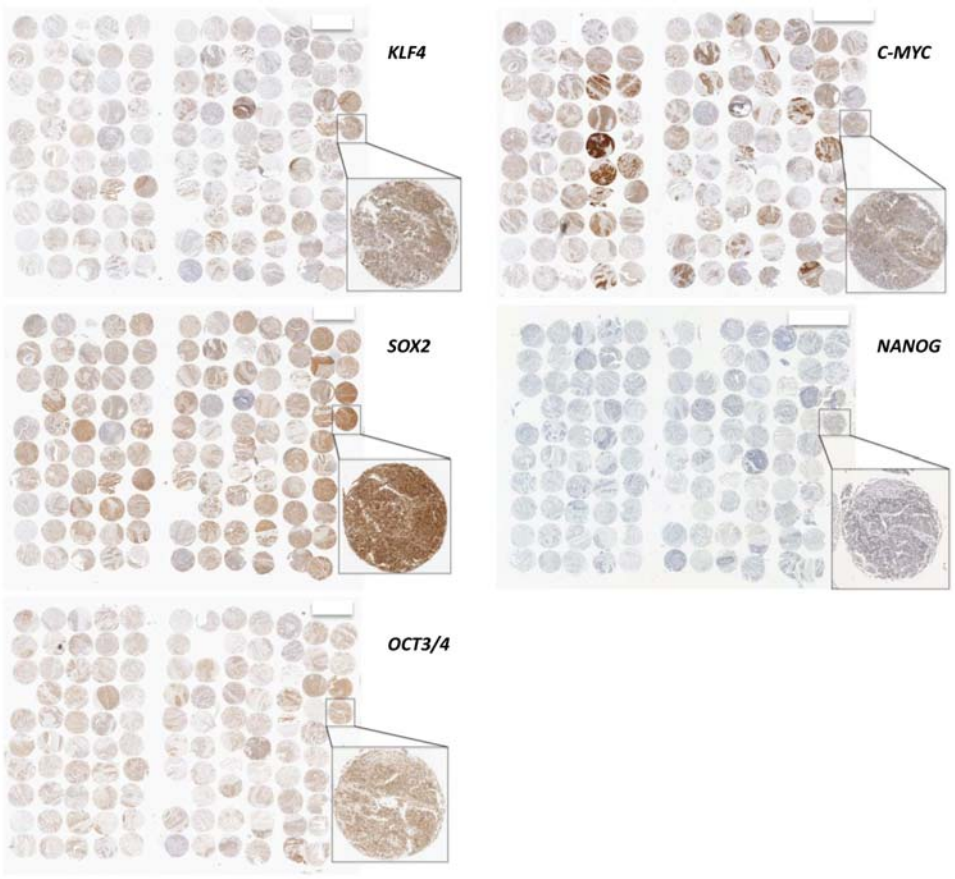

Figure 1. *SOX2, OCT3/4. KLF4, c-MYC and NANOG expression in tissue microarray. All genes stained not only in the cytoplasm but also in the nuclei. Magnified areas shown on the right represent positive staining for each gene.*

two groups, which were a low expression group (total score of 0-3) or a high expression group (total score of 4 or 5). Regarding the expression of *NANOG*, a score of 3 or more was defined as positive according to the distribution of the sum total score.

Statistical analysis. The chi-square test, Fisher's exact test and *t*-test were used to compare clinicopathological data. The overall survival (OS) rate and the cause-specific survival (CSS) rate after surgery were calculated for each group by the Kaplan–Meier method, and differences were assessed by the Log-rank test and Wilcoxon test. For evaluation of cluster analysis, the Wald method was used. A *p*-value less than 0.05 was assumed to indicate significance. All analyses were carried out with JMP 9.0 software (SAS Institute Inc, North Carolina, USA).

Results

Of 81 spots, 80 spots were evaluable for *KLF4*, *SOX2* and *c-MYC*, 79 spots for OCT3/4, and 76 spots were evaluable for *NANOG* because some spots did not have enough cancer cells for evaluation or they peeled off during the staining procedure. As a result of immunohistochemical staining with antibodies to *c-MYC*, *NANOG*, and *SOX2*, nuclei were stained mainly and a low amount of cytoplasm was also stained. The nuclei and the cytoplasm were stained to the same degree on immunohistochemical staining with antibodies to *KLF4* and *OCT3/4* (Figure 1).

Tumors from 34 patients (44.7%) were positive for *NANOG*, 30 (37.5%) for *KLF4*, 42 (52.5%) for *c-MYC*, 22 (27.5%) for *SOX2* and 24 (30.4%) for *OCT3/4* (Table I). The expression of these five genes was not associated with the depth of tumor, lymph node metastasis or pathological stage.

KLF4 expression was associated with histological types; however, other genes were not. Although the overall prognosis of the patients was not associated with these five genes, the cause-specific prognosis of the patients with tumor with high expression of *NANOG* was significantly better than that of those with low expression of *NANOG* (p=0.043) (Figure 2). Furthermore, *NANOG* expression was an independent prognostic factor regarding cause-specific prognosis (risk ratio=0.5, p=0.031) (Table II). Patients with *c-MYC*-positive tumor tended to have a worse prognosis than

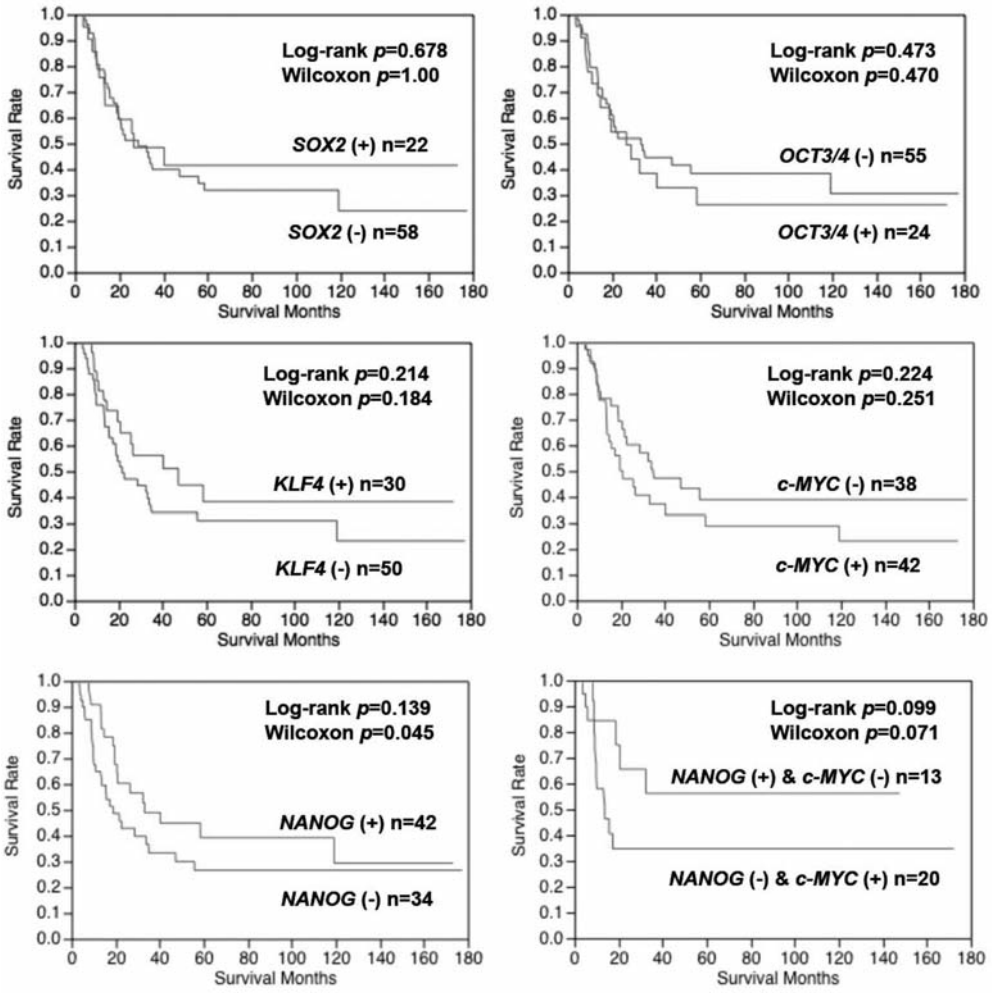

Figure 2. *Cause-specific survival curves of the patients regarding expression of SOX2, OCT3/4. KLF4, c-MYC and NANOG. The prognosis of the patients with a high expression of NANOG was significantly better than that of those with a low expression of NANOG (p=0.043, generalized Wilcoxon test).*

those with *c-MYC*-negative tumors, however *c-MYC* was not an independent prognostic factor for the patients (Table II). Combination analysis of *NANOG* and *c-MYC* revealed that the prognosis of the patients with *NANOG* positive and *c-MYC* negative tumors were better than that of those with *NANOG*-negative and *c-MYC*-positive tumors, however, there was no statistical significance (Figure 2).

With regard to chemosensitivity, patients with *NANOG*-positive tumors had significantly better prognosis than those with *NANOG*-negative tumors among the patients who underwent postoperative cisplatin-based chemotherapy (Figure 3A). Patients with *c-MYC*-positive tumors also tended to have a worse prognosis than those with *c-MYC*-negative tumors (Figure 3B). On the other hand, among the patients without postoperative chemotherapy, the prognosis of the patients did not differ between those with *NANOG* positive and those with *NANOG* negative tumors (Figure 3C), nor between those with *c-MYC*-positive and *c-MYC*-negative tumors. (Figure 3D). The background of the patients with postoperative chemotherapy did not differ depending on NANOG or c-MYC expression (Table III).

In clustering analysis, 75 cases had complete sets of all genetic data and were evaluable for analysis. Clustering analysis with absolute levels of expression of each gene resulted in four clusters (A, B, C and D) (Figure 4A). The patients in cluster C, which represents those with positive expression of *NANOG* low expression of *c-MYC*, and moderate expression of *KLF4*, *SOX2*, and *OCT3/4*, had a better prognosis than patients in other clusters (Figure 4B).

Discussion

iPS were induced from the somatic cells of mice and human fibroblasts by Takahashi and Yamanaka (1). Among the iPS-

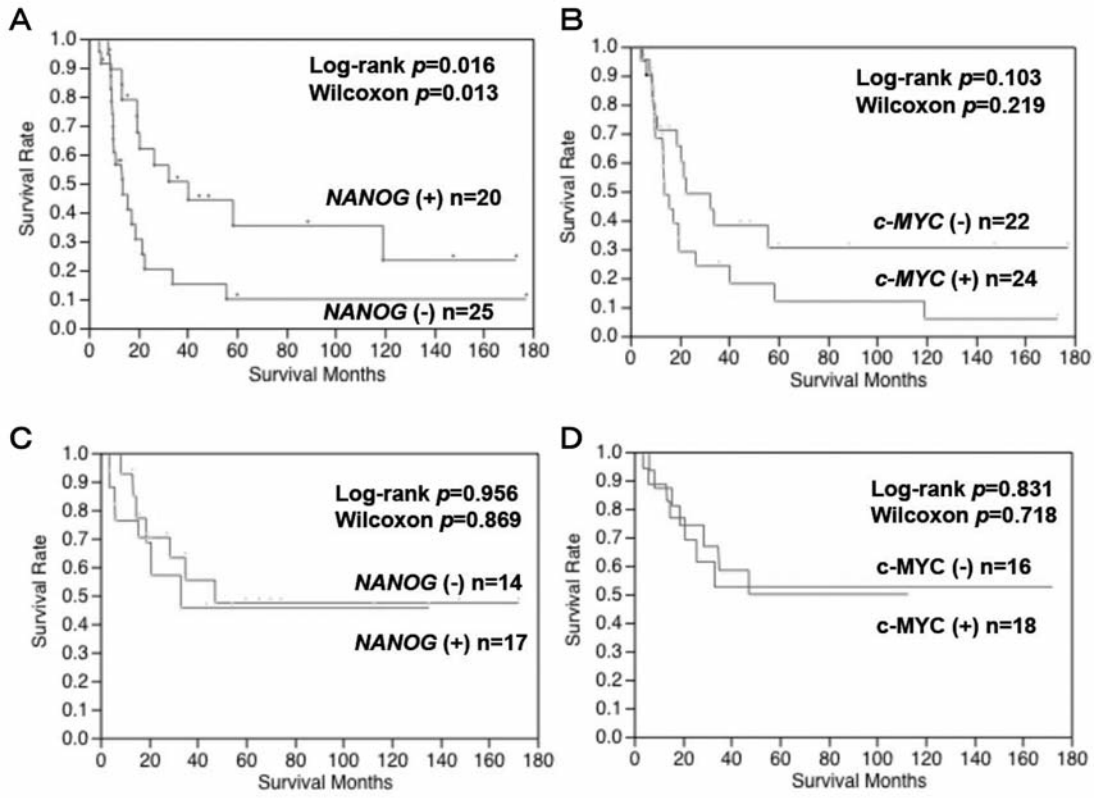

Figure 3. *A and B: Survival curves of the patients who received postoperative cisplatin based chemotherapy according to tumor expression of NANOG and c-MYC. Patients with NANOG-positive tumors had a significantly better prognosis than those with NANOG-negative tumors. C and D: Survival curves of the patients who did not receive postoperative cisplatin-based chemotherapy according to tumor expression of NANOG and c-MYC. The prognosis of the patients did not differ between the patients with NANOG-positive and NANOG-negative expression.*

Table II. *Cox multivariate analysis of the patient data.*

Results of *NANOG* analysis				Results of *c-MYC* analysis			
Term	Risk ratio	95% CI	*p*-Value	Term	Risk ratio	95% CI	*p*-Value
Age >65 years	2.09	1.00-4.39	0.0496	Age >65 years	2.52	1.23-5.18	0.011
Gender (male)	2.12	0.78-7.48	0.152	Gender (male)	2.11	0.77-7.48	0.153
T	2.61	1.19-6.23	0.015	T	2.68	1.27-6.16	0.009
N	1.46	0.61-3.70	0.4	N	1.31	0.58-3.15	0.523
M	1.76	0.64-4.40	0.256	M	2.78	1.09-6.68	0.034
Adjuvant chemotherapy (yes)	1.55	0.70-3.52	0.28	Adjuvant chemotherapy (yes)	1.75	0.83-3.76	0.139
NANOG expression (yes)	0.52	0.27-0.97	0.041	*c-MYC* expression (yes)	1.46	0.80-2.70	0.215

inducing factors (*OCT3/4, SOX2, KLF4, c-MYC, NANOG*, and *Lin28*), *NANOG* is known to control the differentiation of embryonic stem (ES) cells, and it also has a role in maintaining self-repopulating ability (1).

Recently, Du *et al*. reported that the occurrence of *NANOG* expression was positively correlated with the TNM stages and the histopathological differentiation of esophageal SCC tumors ($p<0.01$) (6). However, they did not mention the outcome of the patients. Contrary to this report, our results did not show any association between the TNM stage and histopathological differentiation. Our results also suggest that positive expression of *NANOG* is associated with a favorable

Table III. *The background of the patients treated with and without postoperative cisplatin-based chemotherapy.*

	Postoperative chemotherapy			Without postoperative chemotherapy			Postoperative chemotherapy			Without postoperative chemotherapy		
	NANOG			NANOG			c-MYC			c-MYC		
	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value	(–)	(+)	*p*-Value
Age												
>65	8	7	0.832	13	10	0.75	7	9	0.686	12	12	0.593
<65	17	13		4	4		15	15		4	6	
Gender												
Male	24	19	0.872	13	11	0.889	21	23	0.95	11	15	0.317
Female	1	1		4	3		1	1		5	3	
T												
T1	2	3	0.687	9	2	0.121	3	2	0.524	6	7	0.632
T2	6	3		2	2		6	3		3	1	
T3	15	11		3	7		11	16		5	6	
T4	2	3		3	3		2	3		2	4	
N												
N0	5	5	0.686	8	6	0.815	6	4	0.384	9	8	0.492
N1	20	15		9	8		16	20		7	10	
M												
M0	21	17	0.927	17	14		19	19	0.52	16	18	
M1	4	3		0	0		3	5		0	0	
TNM stage												
1	1	0	0.837	6	2	0.195	1	0	0.571	5	5	0.215
2a	4	5		2	2		5	4		4	1	
2b	3	3		4	1		4	2		3	2	
3	13	9		5	9		9	13		4	10	
4a	4	3		0	0		3	5		0	0	
Histology												
Well/mod	20	16	1	14	12	0.8	18	19	0.821	14	14	0.458
Poor	5	4		3	2		4	5		2	4	

prognosis of patients with esophageal cancer. Furthermore, *NANOG* expression was an independent prognostic factor for patients with esophageal cancer.

With regard to chemosensitivity, Du *et al*. also reported that *NANOG* siRNA enhanced the sensitivity to cisplatin chemotherapy in esophageal cancer in an *in vitro* study (6). Contrary to this report, our results clearly suggest that among the patients who underwent postoperative cisplatin-based chemotherapy, the prognosis of these with the *NANOG*-positive tumors was better than that of those with *NANOG*-negative tumors. Miyoshi *et al*. reported that the transcription factors *OCT3/4, SOX2, KLF4*, and *c-MYC* significantly induced up-regulation of *NANOG* mRNA in four cancer cell lines (7). They also suggested that retroviral-mediated introduction of iPS confers higher sensitivity to chemotherapeutic agents and differentiation-inducing treatment. Taken together, these data indicate that *NANOG* may be a good predictive marker for efficacy of cisplatin-based chemotherapy in esophageal cancer.

c-MYC gene is an important member of the *MYC* gene family, and can translocate and regulate a variety of substances, enable unlimited cell proliferation, and immortalize cells, and is involved in tumor development. Wang *et al*. suggested that positive c-MYC expression was significantly correlated with invasion depth and lymph node metastasis in esophageal SCC (8). They also suggested that the patients with positive expression of *c-MYC* had a significantly poorer prognosis than those without expression. Yang *et al*. reported that down-regulation of c-MYC effectively overcame *AURORA-A*-induced resistance to cisplatin in esophageal cancer cells (9). Although we did not find any significant difference, our results also suggest that c-MYC expression might have prognostic impact in esophageal SCC and *c-MYC* expression might be a predictor of resistance to cisplatin-based chemotherapy.

SOX2 is a high-mobility group box embryonic stem cell transcription factor that is expressed in the developing foregut and normal gastric epithelium and is down-regulated in intestinal metaplasia of the stomach and esophagus. Mendelson *et al*. reported that in a normal esophagus, *OCT3/4*-positive cells are located in the basal layer,

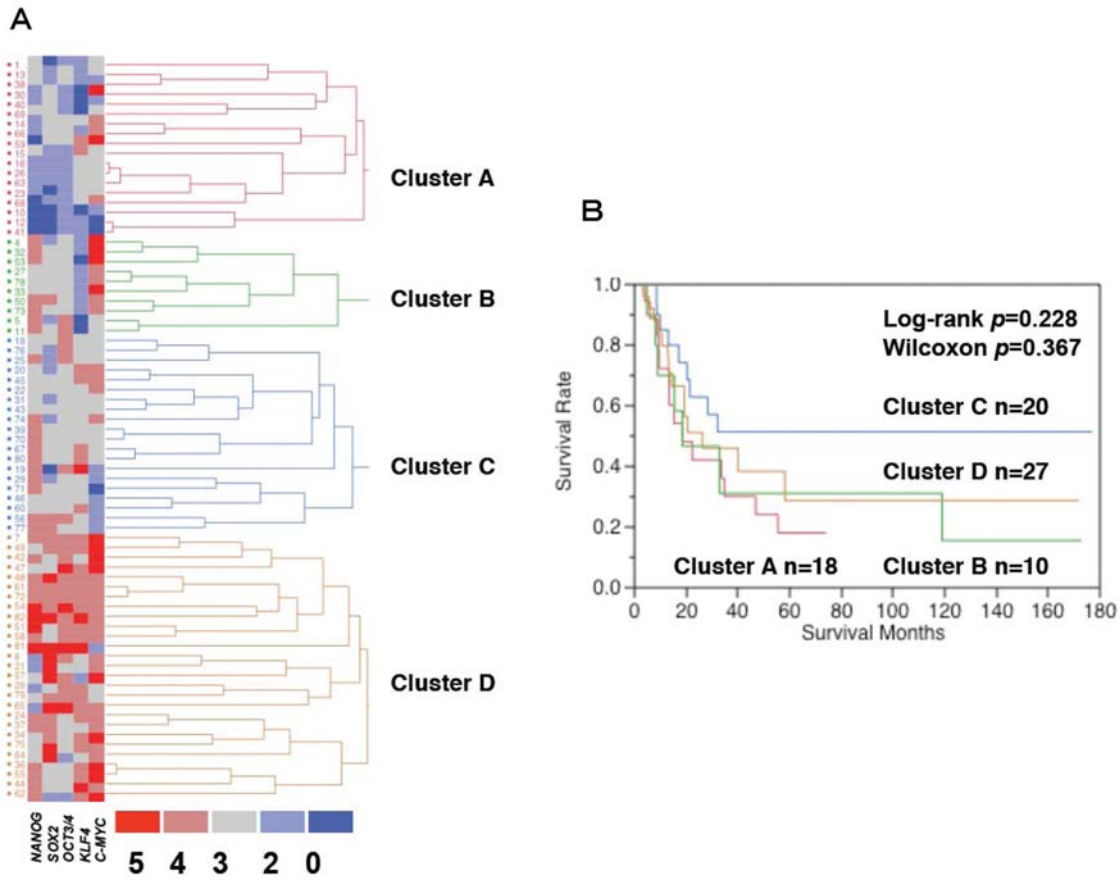

Figure 4. *Clustering analysis and the prognosis of the patients using absolute expression levels of five genes. Cluster C represents the patients with a relatively high expression of NANOG, moderate expression of SOX2, OCT3/4 and KLF4, and low expression of c-MYC (A). Although there was no significant difference among the four groups, cluster C had better prognosis than other clusters (B).*

representing a pool of progenitor cells (10). Thus both *SOX2* and *OCT3/4* are thought to be essential for proliferation in esophageal epithelia. Long and Harnick suggested that *SOX2* is preferentially expressed in sSCC of the esophagus and anal canal compared to adenocarcinomas from these sites (11). Bass *et al*. performed fluorescence *in situ* hybridization (FISH) on tissue microarrays from 63 independent primary esophageal SCC samples and noted amplifications (3q26.33) in seven out of 63 cases, confirming the presence of recurrent amplifications in primary tumors (12). They found that *SOX2* was a main target gene in 3q26.33 and suggested that Sox2-driven tumors show expression of markers for both squamous differentiation and pluripotency. Gen *et al*. reported that up-regulated expression of *SOX2* was associated with poor differentiation of esophageal SCC (13). These studies suggest that *SOX2* may have crucial role in carcinogenesis of SCC.

With regard to the prognostic impact of *SOX2* and *OCT3/4*, in a series of 162 consecutive patients with esophageal SCC, Wang *et al*. showed that 17.9% and 22.8% of the tumors highly expressed *OCT3/4* and *SOX2* proteins, respectively. They also suggested that the expression of these two factors was significantly associated with higher histological grade and poorer clinical survival (14). However, our data did not show any association between clinicopathological factors of the patients and survival. In lung cancer, Wilbertz *et al*. suggested that *SOX2* gene amplification and protein overexpression are associated with a better outcome in squamous cell lung cancer (15). Thus, the prognostic impact of *SOX2* in SCC is still controversial.

KLF4, a member of the KLF family of transcription factors, plays a key role in proliferation, differentiation, and carcinogenesis in a number of gastrointestinal tissues. *KLF4* has distinct functions in esophageal carcinogenesis in mouse models (16). Ectopic expression of *KLF4* inhibits survival and invasion (17, 18). Although our results did not show a significant impact on prognosis, *KLF4* might have favorable prognostic impact on the patients with esophageal SCC. Furthermore, our results suggest that *KLF4* was associated with histological type. Thus *KLF4* might be related to carcinogenesis of esophageal SCC.

Clustering analysis revealed that patients in the cluster with *NANOG*-positive, c-MYC-negative and moderate expression of other iPS genes had better survival compared to those of the other clusters. Thus, in esophageal cancer, *NANOG* and *c-MYC* may have a higher prognostic impact than *SOX2*, *OCT3/4* and *KLF4*.

Finally, it has been well known that due to tumor heterogeneity, a single section from a tumor is not always representative of all the characteristics of the tumor. Although our tissue microarray used one section from the tumor, a careful selection by a skillful pathologist may reduce such a weakness. Thus, we believe that our study may introduce helpful information to understanding the esophageal SCC.

In conclusion, the expression of various iPS inductive factors (*c-MYC, KLF4, OCT3/4, SOX2* and *NANOG*) was detected in esophageal cancer specimens. *NANOG* expression was associated with favorable prognosis in patients with esophageal cancer, whereas *c-MYC* expression might indicate a worse prognosis in patients with esophageal cancer. Furthermore, *NANOG* may be a marker of cisplatin sensitivity and *c-MYC* a marker of cisplatin resistance.

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