

Keratinization of Lung Squamous Cell Carcinoma Is Associated with Poor Clinical Outcome

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Background: Although the World Health Organization (WHO) classification of lung squamous cell carcinoma (SCC) was revised in 2015, its clinical implications for lung SCC subsets remain unclear. We investigated whether the morphologic characteristics of lung SCC, including keratinization, were associated with clinical parameters and clinical outcome of patients.

Methods: A total of 81 patients who underwent curative surgical resection of diagnosed lung SCC, were enrolled in this study. Attributes such as keratinization, tumor budding, single cell invasion, and nuclear size within the tumor, as well as immunohistochemistry of Bcl-xL and pS6 expressions, were evaluated.

Results: The keratinizing and nonkeratinizing subtypes did not differ with respect to age, sex, TNM stage, and morphologic parameters such as nuclear diameter, tumor budding, and single cell invasion at the tumor edge. Most patients with the keratinizing subtype (98.0%) had a history of smoking, whereas the nonkeratinizing group had a relatively higher proportion of never-smokers relative to the keratinizing group (24.0% vs. 2.0%; $p=0.008$, chi-square test). Expression of pS6 (a surrogate marker of mammalian target of rapamycin complex 1 [mTORC1] signaling that regulates keratinocyte differentiation), and Bcl-xL (a key anti-apoptotic molecule that may inhibit keratinization), did not correlate significantly with the presence of keratinization. Patients with the keratinizing subtype had a significantly shorter overall survival (85.2 months vs. 135.7 months, $p=0.010$, log-rank test), and a multivariate analysis showed that keratinization was an independent, poor prognostic factor (hazard ratio, 2.389; 95% confidence interval, 1.090–5.233; $p=0.030$).

Conclusion: In lung SCC, keratinization is associated with a poor prognosis, and might be associated with smoking.

Keywords: Lung; Carcinoma, Squamous Cell; Bcl-X Protein

Introduction

The newly published 2015 World Health Organization (WHO) classification of lung tumors reclassified squamous cell carcinomas (SCC) into keratinizing, nonkeratinizing, and basaloid subtypes¹, similar to the 2005 Head and Neck WHO Classification of nasopharyngeal carcinomas. Typically, keratinization implies lung SCC, although in the absence of unequivocal keratinization, immunohistochemistry is usually required to distinguish nonkeratinizing SCC from adenocarcinoma. Similar to the head and neck cancer classification, the new lung SCC classification was upgraded to address these pathological issues². However, the prognostic or other clinical significance of this new lung SCC subtype classification is unclear, although recent studies of head and neck cancer have

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revealed that compared to the non-keratinizing subtype, the keratinizing subtype is associated with a poorer prognosis^{3,4}. In contrast, studies of the relationship between the keratinizing subtype and prognosis in lung SCC are rare, and one such study reported that the presence of keratinization was not a significant prognostic factor⁵.

According to previous literature, keratinization is accompanied by apoptosis and is ultimately associated with tumor progression in patients with esophageal SCC⁶. The expression of B-cell lymphoma (Bcl)-xL, an oncoprotein involved in lung SCC tumorigenesis, is known to correlate with apoptosis⁷⁻⁹; furthermore, deactivation of the tumorigenic mammalian target of rapamycin (mTOR) signaling pathway, which plays a key role in regulating cellular proliferation, survival, and angiogenesis, also affects apoptosis in lung cancers¹⁰⁻¹³. These key apoptotic factors might correlate with keratinization and thus might affect prognosis. However, potential direct correlations of keratinization with the mTOR pathway and Bcl-xL expression have not been studied in lung SCC.

In the present study, we aimed to characterize the keratinizing and nonkeratinizing subtypes of lung SCC and confirm the effects of keratinization on overall survival (OS). In addition, we aimed to investigate correlations of keratinization with mTOR pathway activation and Bcl-xL expression.

Materials and Methods

1. Patients

Eighty-one patients who underwent surgical treatment of lung SCC between 1993 and 2016 were randomly selected from the Severance Hospital (Seoul, Korea) lung cancer database. To obtain clinical data, we retrospectively reviewed the patients' electronic medical records. Tumor stage was re-evaluated according to the seventh edition of the American Joint Committee on Cancer TNM Staging Manual¹⁴. This study was approved by the Institutional Review Board (IRB) of Severance Hospital (No. 3-2016-0019).

2. Histologic evaluation

All tissue slides were subjected to hematoxylin and eosin staining and evaluated for the presence of the following recently identified poor prognostic factors: tumor budding, single cell invasion, and large nuclei⁵. Initially, the entire tumor set was scanned at $\times 100$ magnification and subjected to a detailed review. First, tumor budding, or the presence of small tumor nests comprising fewer than five tumor cells, were counted in 10 high-power fields (HPFs) at $\times 200$ magnification. We defined a high grade of tumor budding as more than eight tumor budding events per 10 HPFs (Figure 1A, B). Single cell

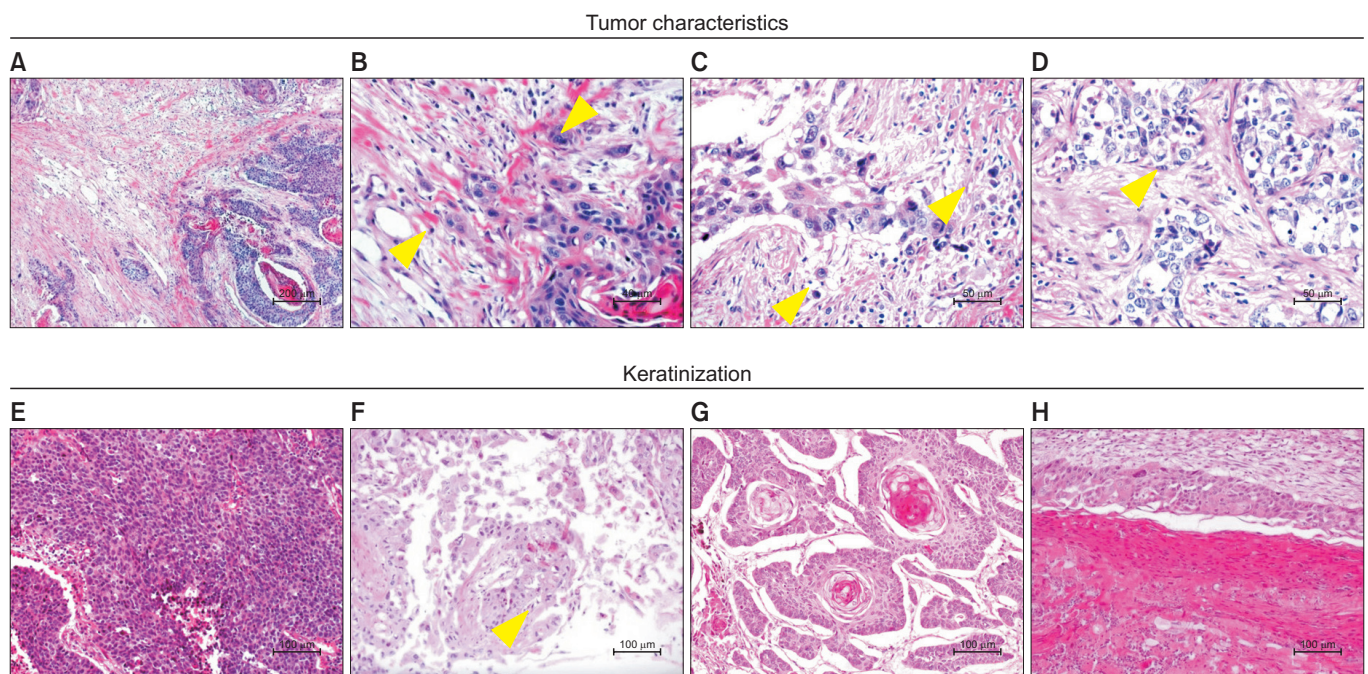


Figure 1. Histologic parameters and patterns of keratinization applied in this study. Tumor budding is observed along tumor edge (A); this is defined as the presence of structures comprising fewer than five tumor cells (arrowheads) at higher magnification (B). Single cell invasion (C) and large nuclei (cancer cell nucleus >4 times than that of a small lymphocyte) (D), nonkeratinization (entire tumor area, $<5\%$ keratinization) (E), cytoplasmic keratinization (F), keratin pearl (G), and layered keratinization (H) (H&E stain; A, $\times 40$; B–D, $\times 400$; E–H, $\times 200$).

invasion was also evaluated at $\times 200$ magnification (Figure 1C), and nuclear features were assessed at $\times 400$ magnification. We calculated the average nuclear diameter of at least 100 tumor cells in at least three HPFs per sample. A large nucleus was defined as a diameter greater than that of four small lymphocytes (Figure 1D).

The keratinization grade was determined, and tumors were classified accordingly as the keratinizing subtype, defined as a keratinizing pattern comprising $\geq 5\%$ of the entire tumor, or the nonkeratinizing subtype, defined as a keratinizing pattern comprising $< 5\%$ of the tumor (Figure 1E). Subsequently, the keratinization grades were refined to nonkeratinization ($< 5\%$) and low (5%–20%), moderate (20%–50%), or severe ($> 50\%$ of the entire tumor) keratinization, similar to the classification used for head and neck cancers¹⁵. The keratinization patterns included cytoplasmic keratinization (Figure 1F), keratin pearl (Figure 1G), and layered keratinization (Figure 1H). All histologic parameters were evaluated independently by a pathologist (Y.J.C.).

3. Immunohistochemistry

To evaluate mTOR complex 1 (mTORC1) and Bcl-xL expression, immunohistochemistry (IHC) staining for pS6 and Bcl-xL proteins was performed using an EnVision+ system (Dako Corp., Carpinteria, CA, USA) according to the manufacturer's instructions. Briefly, sections were deparaffinized, rehydrated, and subjected to antigen retrieval via microwave heating for 10 minutes. Sections were then immersed in a H_2O_2 -phosphate-buffered saline solution prior to overnight incubation with primary anti-pS6 (1:400, Cell Signaling Technology, Danvers, MA, USA) or Bcl-xL antibodies (1:600, Cell Signaling Technology). Subsequently, the sections were incubated with a peroxidase-labeled polymer for 1 hour at 4°C. IHC staining was scored independently at $\times 200$ magnification by Y.J.C. and S.H.K., who were blinded to the clinicopathological data. A semiquantitative evaluation of pS6 and Bcl-xL was performed according to the method described in a previous study¹⁶. The staining intensity was classified as 0 (negative), 1 (trace), 2 (moderate), or 3 (strong), and the frequency of positive cells was classified as 0 ($< 10\%$), 1 (10%–50%), 2 (50%–80%), or 3 ($> 80\%$). The expression score was determined as the product of the staining intensity and frequency of positive cells.

4. Statistical analysis

Associations between categorical variables were analyzed using the chi-square test; this test was also used to assess the linear correlation trend of the keratinization grade with IHC scoring. Disease-free survival (DFS) and OS were estimated using the Kaplan-Meier method, and associations between factors and survival outcomes (OS) were analyzed using the

log-rank test. The Cox proportional hazards model was used for multivariate analyses. SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses, and significance was defined as a p-value of < 0.05 .

Results

1. Smoking correlates with the keratinization of lung SCC

Among the 81 patients enrolled in this study, 56 (69.1%) and 25 (30.9%) were classified as having the keratinizing and nonkeratinizing subtypes of lung SCC, respectively, as confirmed by a pathologic review of tumor slides. The average age of the subjects was 62.8 ± 8.7 years (mean \pm standard deviation); 77 (95.1%) were male, and the majority had stage T2 or T3 disease (84.0%). Among patients with the keratinizing subtype, 21 (37.5%), 19 (33.9%), and 16 patients (28.6%) were classified as having low, moderate, and severe keratinization, respectively. The keratinizing and nonkeratinizing groups did not differ significantly with respect to the distributions of age, sex, T-, N-, pStage, type of surgery, and adjuvant chemotherapy. However, most patients with the keratinizing subtype (98.0%) had a history of smoking, whereas never smokers (24.0%) were significantly more prevalent in the nonkeratinizing subtype group (Pearson's $R=0.262$, $p=0.008$, chi-square test). In a subgroup analysis of smokers, keratinization was more frequently observed among heavy smokers (i.e., ≥ 30 pack-year history of smoking) ($p=0.012$, chi-square test). This finding suggests that keratinization of lung SCC might be related to smoking (Table 1). Other morphologic parameters, such as nuclear diameter, tumor budding, and single cell invasion at the tumor edge, were not found to correlate with the keratinization of lung SCC.

2. mTORC1 and Bcl-xL expression did not correlate with lung SCC keratinization

Because mTOR signaling is known to regulate keratinocyte differentiation, we reviewed the relationship between keratinization and mTORC1 in 51 available lung SCC tissues, using pS6 expression as a surrogate marker. Among these 51 cases, five (9.8%), 12 (23.5%), and 23 (45.1%) exhibited strong, moderate, and trace expression, respectively. An additional 11 cases (21.6%) were negative for pS6. Viable tumors expressed pS6 beneath the shedding area, where keratinization initiates, regardless of keratinization subtype (Figure 2). However, the correlation between keratinization grade and pS6 expression failed to reach statistical significance ($p=0.153$, chi-square test).

In tumors, keratinization is generated by the epithelial layer and is subsequently shed from the tumor margin by apopto-

Table 1. Demographics and pathologic characteristics of patients with and without keratinization

Variable	Nonkeratinization (n=25)	Keratinization (n=56)	Total (n=81)	p-value
Age, yr				0.807
≤65	16 (64.0)	33 (58.9)	49 (60.5)	
>65	9 (36.0)	23 (41.1)	32 (39.5)	
Sex				0.583
Male	23 (92.0)	54 (96.4)	77 (95.1)	
Female	2 (8.0)	2 (3.6)	4 (4.9)	
Smoking status				0.008
Never smoker	6 (24.0)	1 (2.0)	7 (8.6)	
Former smoker	7 (28.0)	18 (35.3)	25 (30.9)	
Current smoker	12 (48.0)	32 (62.7)	44 (54.3)	
Unknown	-	-	5 (6.2)	
Smoking pack-years				0.012
<30	12 (48.0)	9 (17.6)	21 (27.6)	
≥30	13 (52.0)	42 (82.4)	55 (72.4)	
T classification				0.605
T1	4 (16.0)	4 (7.1)	8 (9.9)	
T2	15 (60.0)	39 (69.6)	54 (66.7)	
T3	4 (16.0)	10 (17.9)	14 (17.3)	
T4	2 (8.0)	3 (5.4)	5 (6.2)	
N classification				0.811
N0	16 (64.0)	35 (62.5)	51 (63.0)	
N1	5 (20.0)	9 (16.1)	14 (17.3)	
N2	4 (16.0)	12 (21.4)	16 (19.8)	
pStage				0.938
pStage I	9 (36.0)	22 (39.3)	31 (38.3)	
pStage II	9 (36.0)	18 (32.1)	27 (33.3)	
pStage III	7 (28.0)	16 (28.6)	23 (28.4)	
Type of surgery				0.806
Lobectomy	16 (64.0)	32 (58.2)	48 (60.0)	
Pneumonectomy	9 (36.0)	23 (41.8)	32 (40.0)	
Adjuvant chemotherapy				>0.999
No	15 (60.0)	33 (58.9)	48 (59.3)	
Yes	10 (40.0)	23 (41.1)	33 (40.7)	
Nuclear diameter				0.111
Small	6 (31.6)	31 (55.4)	37 (49.3)	
Large	13 (68.4)	25 (44.6)	38 (50.7)	
Tumor budding				0.493
Low (<8/10 HPFs)	18 (90.0)	44 (80.0)	62 (82.7)	
High (≥8/10 HPFs)	2 (10.0)	11 (20.0)	13 (17.3)	
Single cell invasion				>0.999
Absent	14 (73.7)	40 (72.7)	54 (73.0)	
Present	5 (26.3)	15 (27.3)	20 (27.0)	

Values are presented as number (%).
HPFs: high-power fields.

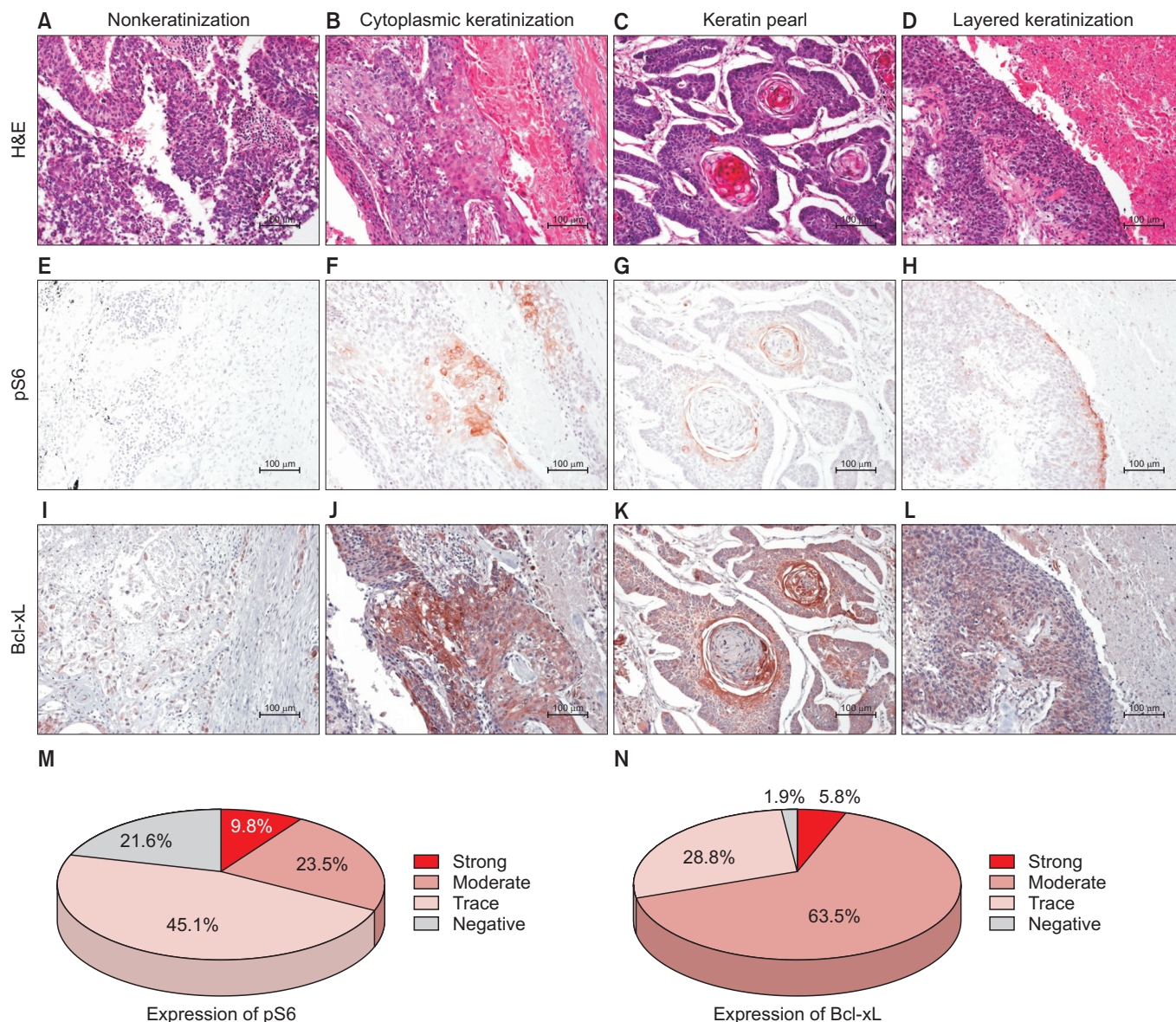


Figure 2. Relationship of keratinization (A–D, H&E stain, ×200) with the expression of pS6 ribosomal protein (Ser235/236; E–H, ×200) and Bcl-xL (I–L, ×200). Images represent nonkeratinization (A, E, I), cytoplasmic keratinization (B, F, J), keratin pearl (C, G, K), and layered keratinization (D, H, L). Distribution of pS6 (M) and Bcl-xL (N) expression in lung squamous cell carcinoma.

sis. Under a hypothesis that Bcl-xL, a key anti-apoptotic molecule, might inhibit keratinization, we investigated the relationship between keratinization and apoptosis in the keratinized area. Although we observed a negative correlation between Bcl-xL and tumor cell keratinization, this relationship did not reach statistical significance (Pearson’s $R = -0.264$, $p = 0.062$, chi-square test) (Figure 2).

3. Keratinization was associated with a poor clinical outcome in lung SCC

We next analyzed the clinical outcomes of lung SCC according to keratinization. Although the keratinizing subtype was associated with a reduced DFS, this difference did not reach statistical significance (119.6 months vs. 122.7 months, $p = 0.459$, log-rank test). However, the keratinizing subtype was associated with significantly shorter OS relative to the nonkeratinizing subtype (85.2 months vs. 135.7 months, $p = 0.010$, log-rank test) (Figure 3).

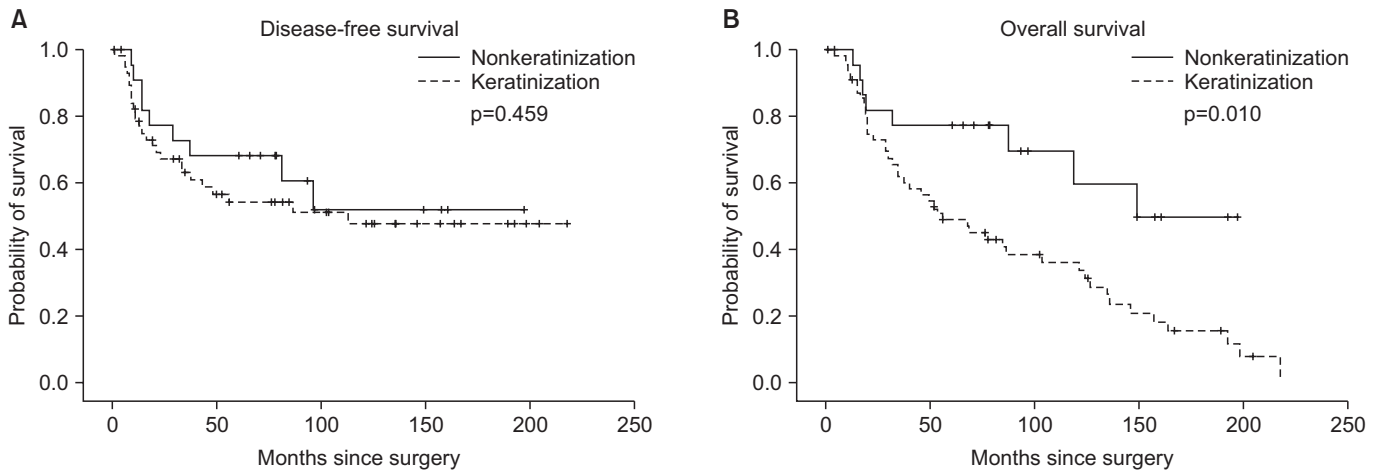


Figure 3. Disease-free survival (A) and overall survival (B) of patients with lung squamous cell carcinoma according to the keratinization status (p-values were obtained using the log-rank test).

Table 2. Univariate and multivariate analyses of overall survival

Variable	Univariate analysis			Multivariate analysis			
	HR	95% CI	p-value	HR	95% CI	p-value	
Age, yr	≤65	1		1			
	>65	1.799	1.031–3.319	0.039	2.154	1.150–4.035	0.017
Sex	Male	1		1			
	Female	2.717	0.375–19.707	0.323	3.879	0.107–140.535	0.459
Smoking pack-year	Never	1		1			
	Former	8.824	1.157–67.285	0.036	5.661	0.713–44.969	0.101
	Current	6.062	0.825–44.534	0.077	3.729	0.491–28.310	0.203
pStage	I	1		1			
	II	1.139	0.590–2.202	0.698	1.438	0.713–2.899	0.310
	III	1.839	0.936–3.613	0.077	2.558	1.207–5.422	0.014
Adjuvant chemotherapy	No	1					
	Yes	1.669	0.951–2.929	0.074			
Nuclear diameter	Small	1					
	Large	1.231	0.701–2.163	0.469			
Tumor budding	Low (<8/10 HPFs)	1					
	High (≥8/10 HPFs)	2.052	1.043–4.039	0.037			
Single cell invasion	Absent	1					
	Present	2.178	1.190–3.987	0.006			
Keratinization grade	Nonkeratinization	1		1			
	Keratinization	2.597	1.219–5.531	0.013	2.389	1.090–5.233	0.030

HR: hazard ratio; CI: confidence interval; HPFs: high-power fields.

In a univariate analysis, old age (p=0.039) and a history of smoking (p=0.036) were associated with a significantly shorter OS. Among histologic parameters, a high tumor budding

grade (p=0.037), presence of single cell invasion (p=0.006), and keratinizing subtype (p=0.013) were associated with a significantly poorer OS. To confirm keratinization as an inde-

pendent prognostic factor for OS in lung SCC, we performed a multivariate analysis that included age, sex, smoking status, tumor stage, and keratinization. This analysis identified older age (>65 years; hazard ratio [HR], 2.154; 95% confidence interval [CI], 1.150–4.035; $p=0.017$), advanced stage (stage III; HR, 2.558; 95% CI, 1.207–5.422; $p=0.014$), and keratinization (HR, 2.389; 95% CI, 1.090–5.233; $p=0.030$) as independent poor prognostic factors (Table 2).

Discussion

Keratins are epithelium-specific intermediate filament proteins that play a central role in maintaining the structural integrity of cells and are thought to be involved in cell differentiation. Keratinocytes follow a unique program of terminal differentiation and apoptotic cell death, ultimately leading to the formation of the keratin layer¹⁷. For these reasons, keratinization within tumors is considered a marker of well-differentiated SCC of the head and neck, as well as of the lungs^{18,19}.

Studies on head and neck SCC showed an association between the keratinizing subtype and a poorer OS (compared with the nonkeratinizing subtype)^{4,20,21}. Although the underlying mechanism remains unclear, some researchers have indicated that keratinization is associated with poor prognosis in human papillomavirus-negative head and neck SCC²². Few studies have addressed OS with respect to keratinization in lung SCC. A recent study provided a key finding regarding the effects of keratinization on OS in this cancer type. Specifically, Xiao et al.²³ demonstrated that IKK α is expressed at low and high levels in keratinizing and nonkeratinizing lung SCC, respectively. As a higher level of IKK α gene expression was found to correlate with good OS in patients with lung cancer, keratinizing lung SCC might lead to a poorer OS. Furthermore, among cases of nonkeratinizing lung SCC, the differentiated subtype correlated significantly with higher IKK α expression relative to the undifferentiated subtype; in other words, keratinization of lung SCC might correlate with a poor OS, regardless of the differentiation status²³.

Smoking affects both the immunologic system and keratinization process²⁴; specifically, increased keratinization of the oral cavity is frequently observed in smokers because epithelial cells exposed to cigarette smoke defend themselves via elevated cytokeratin levels^{25–27}. The present study therefore demonstrated similar effects of smoking on keratinization in the lungs.

mTOR and Bcl-xL, which are known to associate with keratinocyte differentiation and apoptosis, respectively, were identified and analyzed in previous studies^{28,29}. mTORC1 not only plays roles in mesenchymal cell differentiation processes such as adipogenesis, osteogenesis, and myogenesis, but is also involved in epithelial cell differentiation and integrity maintenance. Although the correlation between keratinization

and mTORC1 activity (per the surrogate marker pS6) did not reach statistical significance in our study, we observed some overlap of positive pS6 staining with keratinization. Areas of keratinization initiation correlated negatively with the expression of the anti-apoptotic protein Bcl-xL, consistent with previous studies that demonstrated keratinization progression at the point of apoptotic pathway activation⁶. Accordingly, we suggest that apoptosis is inevitable during the process of keratinization; however, additional detailed studies of the underlying mechanisms should be conducted to confirm this hypothesis.

We must note some limitations of the present study. For example, the number of study subjects was small. In addition, these subjects were limited to patients with surgically resectable lung SCC. A large cohort study that includes patients with unresectable lung SCC, as well as molecular profiling, will be needed to identify the detailed underlying mechanisms. Nevertheless, this study demonstrated that keratinization is a significantly poor prognostic factor in lung SCC, thus warranting large-scale studies to confirm this finding.

Herein, we demonstrated the association of keratinization of lung SCC with a poor clinical outcome, in comparison with the nonkeratinization subtype. This outcome might result from the association of keratinization with smoking and the apoptotic features usually observed at the keratinizing site.

Conflicts of Interest

No potential conflicts of interest relevant to this article have been reported.

Acknowledgments

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