Expression of phosphatase and tensin homolog and programmed cell death ligand 1 in adenosquamous carcinoma of the lung

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ABSTRACT

Background: Lung adenosquamous carcinoma (ASC) is a rare variant of non-small cell lung cancer (NSCLC) with poor prognosis. Certain biological differences may exist between these tumors and other common histological types of NSCLC, including adenocarcinoma (ADC) and squamous cell carcinoma (SCC). The phosphoinositide 3-kinase (PI3K) pathway, which links oncogenes and multiple receptor classes to essential cellular functions, is activated by phosphatase and tensin homolog (PTEN) loss. The PTEN loss has been suggested to induce programmed cell death ligand 1 (PD-L1) expression in various cancer types.

Objective: Here, we sought to determine the relationships between the expression of PTEN and PD-L1 in each component of ASC with ADC and SCC, and clinical parameters.

Material and methods: Tissue microarrays of 148 cases of surgically resected lung ADC and 102 cases of SCC, as well as full sections from 28 ASC cases, were analyzed immunohistochemically for the expression of PTEN and PD-L1.

Results: PD-L1 expression was similar between the adenocarcinoma component of ASC vs. lung ADC and between the squamous component of ASC vs. lung SCC. PTEN loss was higher in lung ADC than in the adenocarcinoma component of ASC and significantly higher in lung SCC than in the squamous component of ASC. PD-L1 expression was higher in the squamous component than in the glandular component of the 28 ASC cases, but PTEN loss was similar. Overall, PTEN loss was higher in lung SCC than in lung ADC and both components of ASC. In lung SCC and glandular portions of ASC, PD-L1 expression levels were significantly associated with those of PTEN. The loss of PTEN correlated with smoking status in patients with lung ADC.

Conclusions: Our results implied that both squamous and glandular components of ASC may share the same oncogenic driver pathway for carcinogenesis. However, the squamous cell components of ASC likely escape the immune surveillance better than the glandular components due to higher PD-L1 expression.

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Abbreviations: ASC, adenosquamous carcinoma; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; PD-L1, programmed cell death ligand 1.

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1. Introduction

Lung cancer is the second most commonly diagnosed cancer, and 234,030 new cases of lung cancer are estimated to be diagnosed in the US in 2018 [1]. Numerous studies demonstrate that tyrosine kinase activation contributes to tumor growth and malignant progression in different tissues, including lung cancer [2,3].

The phosphoinositide 3-kinase (PI3K) signaling axis plays important regulatory roles in cell proliferation and differentiation by activating several oncogenes and downstream effector pathways

causing tumorigenesis. It can be activated by the loss of phosphatase and tensin homolog (PTEN), mutations in the genes encoding PI3K, or constitutive activation of upstream regulatory receptor tyrosine kinase pathways [4,5]. Hyperactivation of the PI3K pathway might be involved in lung cancer, and certain expression patterns of PTEN confer prognostic significance [6,7].

Interactions between programmed cell death 1 (PD1) and its ligand (PD-L1) constitute a key immune checkpoint, which maintain self-tolerance and protect peripheral tissues by modulating immune responses [8]. Ligation of PD-L1 on cancer cells to PD1 on T cells suppresses T cell activation and proliferation, inducing T cell apoptosis [9].

Interconnections between oncogenic driver and immunoregulatory pathways in cancer cells have not been fully elucidated, however, there is evidence for a genetic regulation of immune response [10,11]. PTEN can directly regulate interferon (IFN) response signaling pathways and thus, plays a role in immune regulation [12].

In an *in vitro* model of NSCLC, PD-L1 expression correlated with reduced PTEN protein expression [13]. In glioblastoma, it was suggested that PTEN loss was an important mechanism for PD-L1 expression in cell lines and tumor specimens [14]. Similar results were found in breast, prostate, and lung carcinomas, indicating that PD-L1 expression is PI3K pathway-dependent [15–17]. PD-L1 protein (but not mRNA) expression was significantly increased in colorectal cancer cells with reduced PTEN expression [18].

Targeted therapy unrelated to genetic alterations is needed for most lung cancers deficient in actionable divergent genes. The application of the newly-developed immune checkpoint inhibitors has been associated with significant response rates in several tumor types [19]. Additionally, anti-PD1/PD-L1-based monoclonal antibodies have shown higher efficacy as a second-line treatment for patients with advanced NSCLC [20]. Hence, PD1 and PD-L1 are potential targets in lung cancers, and PD-L1 expression may be a biomarker for the therapeutic response to anti-PD1/PD-L1 antibodies [21].

Not all tumor cells express PD-L1. In different tumors, many extrinsic and intrinsic factors regulate PD-L1 expression. One such cellular control mechanism includes the inactivation of the tumor suppressor gene *PTEN* [22,23]. Moreover, resistance to some immune checkpoint inhibitors is partly related to PTEN loss [11,24].

Lung adenosquamous carcinoma (ASC) is an unusual subtype of non-small-cell lung cancer (NSCLC) with worse prognosis than adenocarcinoma (ADC) or squamous cell carcinoma (SCC), and is more aggressive [25]. This observation indicates the existence of certain biological differences among these three histological types of NSCLC [26].

There is no adequate treatment for ASC as deep understanding of its genetic basis and molecular characteristics is lacking. Surgery, platinum-based chemotherapy, and radiotherapy are the primary treatments for ASC. The poor prognosis of ASC patients has prompted a search for more effective treatments, such as targeted therapy. Mutations of the epidermal growth factor receptor (*EGFR*) gene have been detected in ASC of the lung [27], and recent clinical data have shown that *EGFR* tyrosine kinase inhibitors are an effective treatment for such cancers [28]. There has also been a case report of a Japanese patient harboring *EGFR*-activating mutations in ASC after 3 years on gefitinib [29].

Genetic profiling and major driver oncogene alterations in ASC have been detected by different methods. ASC with *EGFR* mutations is the major type of lung ASC. Mutations of the PI3K signaling pathway genes *PIK3CA* and *PTEN* were also found in some cases [30].

A comparative study of PD-L1 expression was performed in SCC and ADC components of ASC and in pure SCC and ADC forms. PD-L1

expression level was similar in the squamous cell component of ASC vs. lung SCC, as well as in the adenocarcinoma component of ASC vs. lung ADC. However, for the first time, it was reported that PD-L1 expression levels differed significantly between the two components of lung ASC [31].

Nonetheless, little is known about the relationship between the expression patterns of PD-L1 and PTEN in ASC. To better understand the biological mechanism underlying the immune and oncogenic nature of ASC, we investigated PD-L1 and PTEN expression levels in ASC, ADC, and SCC, using clinicopathological data.

2. Materials and methods

2.1. Study samples

We collected 50 histologically proven cases of ASC, 167 cases of ADC, and 116 cases of SCC for this study, which was approved by the Institutional Ethics Committee. The clinicopathological information related to age, sex, smoking history, staging, lymphatic, vascular, and pleural invasion, lymph node metastasis and intrapulmonary metastasis was collected from the clinical records.

Some specimens were excluded because of the absence of tumor cells, presence of questionable inflammatory cells, or edge artifacts in immunohistochemistry (IHC). Therefore, for PD-L1 IHC staining, 28 cases of ASC, 133 cases of ADC, and 88 cases of SCC were included. For PTEN IHC staining, 27 cases of ASC, 148 cases of ADC and 102 cases of SCC were scored.

2.2. Slide preparation

For ASC, after preparing full-section hematoxylin and eosin-stained slides from formalin-fixed, paraffin-embedded tissue blocks, each case was reviewed for both squamous and glandular components in at least 10% of the tumor area. These cases were confirmed by a pulmonary pathologist (J.F.) after histological diagnosis based on the World Health Organization criteria [32]. In cases of histologically atypical adenocarcinoma, IHC analyses for napsin A, thyroid transcription factor 1, and the atypical squamous components p40 and CK14 were also employed to confirm the final results. For ADC and SCC, tissue microarray slides containing 0.7-mm cores of lung ADC and SCC cases were used [33].

2.3. IHC analyses and antibody selection

Immunohistochemical analyses of 4- μ m thick tissue sections stained with anti-PD-L1 monoclonal antibody (clone 28-8) and an anti-PTEN antibody (clone D4.3) were carried out using Ventana Bench Mark XT Automated stainer (Ventana Medical Systems, Tucson, AZ, USA) and BOND III fully automated stainer (Leica Biosystems, Melbourne, Australia) following standard protocols.

2.4. Scoring and interpretation of IHC

All stained slides were scanned at 20 $\times$ magnification. The scanned slides were scored by two pathologists (A.M.H. and Y.K.). For PTEN, scoring was based on the intensity and distribution of staining in slide images. The intensity of staining was scored as 0 (no staining), 1 (pale yellow), 2 (clay-colored), or 3 (sepia). The distribution was scored as 0 (0–10%), 1 (11–25%), 2 (26–50%), or 3 (>50%). Next, the sum of the intensity score and distribution score was used to generate the total score (TS) of TS0 (sum $\leq$ 2), TS1 (sum = 3), and TS2 (sum $\geq$ 4). TS0 and TS1 were defined as negative (–) expression, and TS2 was defined as positive (+) expression. The expression levels were considered as membrane or cytoplasmic

components.

PD-L1 expression was scored as the percentage of viable tumor cells exhibiting a complete circumferential or partial linear plasma membrane staining at any intensity in the entire specimen. The percentages were categorized as < 1%, <10%, <25%, <50% and $\geq$ 50%. The total expression in less than 1% or in less than 10% of all viable tumor cells was regarded as negative (–) expression. The remaining categories were scored as positive (+) expression. The staining of immune cells for PD-L1 was not considered as positive and served only as an internal positive control.

2.5. Statistical analyses

Chi-squared and Fisher's contingency tests were used to examine the relationships between the variables. If the *P* value was less than 0.05, the result was considered statistically significant. The SAS software was used for all data analyses.

3. Results

In ASC, the expression levels of PD-L1 and PTEN in the glandular and squamous regions were examined separately (Fig. 1).

3.1. PD-L1 expression levels in lung ASC, ADC, and SCC

When glandular and squamous components of 28 lung ASC samples were examined separately, the levels of PD-L1 expression were 21.43% ($n = 6/28$) and 39.29% ($n = 11/28$), respectively. The difference was nominal and did not reach statistical significance ($P = 0.162$). In lung ADC, PD-L1 expression level was 29.32% ($n = 39/133$), which was similar to that in the glandular component of lung ASC ($P = 0.491$; Table 1). In lung SCC, PD-L1 expression level was 30.68% ($n = 27/88$), which did not differ from that in the squamous component of lung ASC ($P = 0.489$; Table 1).

3.2. PTEN expression levels in lung ASC, ADC, and SCC

The analysis of 27 lung ASC cases revealed that the rate of PTEN loss was similar ($P = >0.99$) between the glandular component, (48.14%, $n = 13/27$) and the squamous component (44.44%, $n = 12/27$). In lung ADC, the rate of PTEN loss (63.51%, $n = 94/148$) exceeded the one in the glandular component of lung ASC ($P = 0.140$; Table 2). In lung SCC, the rate of PTEN loss was 75.49% ($n = 77/102$), which was significantly higher than that of the squamous component of lung ASC ($P = 0.004$; Table 2).

3.3. PTEN expression and PD-L1 status

In SCC, PTEN expression was significant in 13 (50%) of 26 PD-L1-positive cases ($P = 0.005$). Only 12 cases out of 60 PD-L1-negative cases (20%) showed PTEN expression, while the remaining 48 cases (80%) were PTEN-negative (Table 3).

Table 1

Comparison of PD-L1 expression between ADC and glandular component of ASC, and between SCC and squamous component of ASC.

Types of tumor	PD-L1		Total	Expression Rate	<i>P</i>
	(+)	(–)			
ADC	39	94	133	29.32%	0.491
Glandular component of ASC	6	22	28	21.43%	
SCC	27	61	88	30.68%	0.489
Squamous component of ASC	11	17	28	39.29%	

ADC, adenocarcinoma; ASC, adenosquamous cell carcinoma; PD-L1, programmed cell death ligand 1; SCC, squamous cell carcinoma.

Both PTEN and PD-L1 expression levels were significantly associated with the glandular component of ASC ($P = 0.041$) (Table 3). There were no statistically significant associations between PTEN and PD-L1 expression levels in the ADC and squamous components of lung ASC ($P = 0.558$ and $P = >0.99$) (Table 3).

3.4. Relationship between PD-L1 and PTEN expression levels and clinicopathological characteristics

Among the 28 patients with lung ASC, 23 were men, five were women, eight were smokers, four were nonsmokers, and 16 provided no data. The median age at diagnosis was 69.5 years (range: 49–80 years). The tumors were classified as stage I, II, III, and IV in 10 (35.72%), eight (28.57%), three (10.71%), and one (3.57%) case, respectively. No data for staging were available in six (21.43%) cases. Pleural, lymphatic, and vascular invasion, positive node and intrapulmonary metastases were observed in 16 (57.14%), 21 (74%), 20 (71.44%), 10 (35.71%), and 3 (10.71%) cases, respectively. Neither PD-L1 nor PTEN expression levels in the glandular and squamous components showed a significant association with clinicopathological characteristics (Supplementary Table S1).

Among the 148 patients with lung ADCs, 88 were men, 60 were women, 72 were smokers, and 76 were nonsmokers. The median age at diagnosis was 67 years (range: 30–89 years). The tumors were classified as stage I, II, III, and IV in 87 (58.78%), 25 (16.89%), 28 (18.92%), and eight (5.41%) cases, respectively. Pleural, lymphatic, and vascular invasion, positive node and intrapulmonary metastases were observed in 57 (38.51%), 76 (51.35%), 56 (37.84%), 45 (30.41%), and 26 (17.57%) cases, respectively. PD-L1 and PTEN expression levels showed no association with most of the clinicopathological characteristics (Supplementary Table S2): only smoking status correlated significantly with PTEN loss ($P = 0.017$) (Table 4).

Among the 102 SCC patients, 96 were men, six were women, 45 were smokers, 38 were nonsmokers, and 19 provided no data. The median age at diagnosis was 67 years (range: 40–88 years). The tumors were classified as stage I, II, III, and IV in 49 (48.04%), 34 (33.33%), 16 (15.69%), and one (0.98%) case, respectively. No data for staging were available in two (1.96%) cases. Pleural, lymphatic, and

Fig. 1. Representative images of adenosquamous carcinoma at 5x. A Hematoxylin and eosin-stained section (Arrow – glandular component, Star – squamous component). B Positive PD-L1 expression in both glandular and squamous components. C Positive PTEN expression in both glandular and squamous components.

Table 2

Comparison of PTEN expression between ADC and glandular component of ASC, and between SCC and squamous component of ASC.

Types of tumor	PTEN		Total	Loss of expression rate	P
	(+)	(–)			
ADC	54	94	148	63.51%	0.14
Glandular component of ASC	14	13	27	48.14%	
SCC	25	77	102	75.49%	
Squamous component of ASC	15	12	27	44.44%	

ADC, adenocarcinoma; ASC, adenosquamous cell carcinoma; PTEN, phosphatase and tensin homolog; SCC, squamous cell carcinoma.

Table 3

Comparison of PD-L1 and PTEN expression in ADC, ASC, and SCC.

	ADC		Glandular component of ASC		Squamous component of ASC		SCC	
	(n = 127) (P = NS)		(n = 27) (P = 0.041)		(n = 27) (P = NS)		(n = 86) (P = 0.005)	
	PTEN (+)	PTEN (–)	PTEN (+)	PTEN (–)	PTEN (+)	PTEN (–)	PTEN (+)	PTEN (–)
PD-L1(+)	17	22	5	0	6	4	13	13
PD-L1(–)	33	55	9	13	9	8	12	48
Total	50	77	14	13	15	12	25	61

ADC, adenocarcinoma; ASC, adenosquamous cell carcinoma; PD-L1, programmed cell death ligand 1; phosphatase and tensin homolog, PTEN; SCC, squamous cell carcinoma.

Table 4

Relationship between PTEN expression and smoking status in patients with ADC, each component of ASC and SCC.

		PTEN expression							
		ADC		Glandular part of ASC (P = 0.545)		Squamous part of ASC (P = >0.99)		SCC (P = 0.798)	
		(P = 0.017)							
		+	–	+	–	+	–	+	–
Smoking	Present	19	53	3	4	3	4	10	35
	Absent	35	41	3	1	2	2	10	28
	No data	–	–	8	8	10	6	5	14
Total		54	94	14	13	15	12	25	77

PTEN, phosphatase and tensin homolog; ADC, adenocarcinoma; ASC, adenosquamous cell carcinoma; SCC, squamous cell carcinoma.

vascular invasion, positive node and intrapulmonary metastasis were observed in 36 (35.29%), 38 (37.26%), 45 (44.12%), 36 (35.29%), and 19 (18.63%) cases, respectively. There was no significant association between PD-L1 and PTEN expression levels and clinicopathological characteristics in SCC cohort ([Supplementary Table S3](#)).

4. Discussion

The molecular basis of lung cancer is complex and heterogeneous, as it involves the activation of growth-promoting pathways and inhibition of tumor-suppressor pathways [34]. PTEN, a tumor suppressor protein, regulates many cellular processes [35]. In addition to oncogenic pathways, cancer cells use various strategies to escape from immune surveillance [36]. The interaction between PD-L1 and its co-stimulatory signal PD1 induces T cell apoptosis or exhaustion [37]. The relationships between the oncogenic tumor driver pathways and immunoregulatory pathways such as PD-L1 have not been fully elucidated. However, the function of PTEN in the regulation of innate and adaptive immunity has been demonstrated [12]. Thus, we analyzed the expression of PTEN and PD-L1 in ASC and compared their levels to those in more common subtypes of NSCLC.

PD-L1 expression levels were similar between the squamous cell component of ASC and lung SCC (39.29% vs. 30.68%), as well as between the glandular component of ASC and lung ADC (21.43% vs. 29.32%). Therefore, no trend was apparent for differential PD-L1 expression between SCC or ADC and ASC. This finding also suggests that anti-PD-1/PD-L1 monoclonal antibodies are a promising

therapeutic strategy for ASC patients with PD-L1 upregulation.

Our results show that PTEN loss is greater in lung SCC than in lung ADC (75.49% vs. 63.51%). This observation is in agreement with previous observations that low PTEN expression is more prevalent in SCC than in ADC [38,39]. Furthermore, PTEN loss was more significant in SCC than in the squamous component of ASC. Therefore, the biology of lung ASC may be more complex and divergent than that of SCC and ADC.

It has been suggested that lung ADC can progressively transdifferentiate to SCC with pathologically mixed ASC representing the intermediate stage, which might confer drug resistance and worse prognosis [40]. We found higher PD-L1 expression level in the squamous cell component than in the glandular component of ASC (39.29% vs. 21.43%). Consistently, Shi et al. [31] reported positive PD-L1 expression in 38.89% and 11.11% cells of squamous and adenocarcinoma component, respectively, and attributed poorer prognosis of ASC to the escape from immune response resulting from the gain of PD-L1 expression during transdifferentiation.

The results from the screening of the major driver oncogene alterations in lung ASC showed high convergence rates for identical *EGFR*, *K-ras*, and *B-Raf* mutations in both adenomatous and squamous components, suggesting a role for monoclonality in the histogenesis of ASC [41]. In our study, PTEN loss was evident in both ASC components, and the loss rate was virtually the same (44.44% vs. 48.14%), supporting the monoclonal theory of ASC emergence. Thus, both components may share the same oncogenic driver pathway for carcinogenesis.

In tumor cells, two general mechanisms regulate PD-L1 expression: innate immune resistance and adaptive immune

resistance. In innate immune resistance, PD-L1 expression is driven by constitutive oncogenic signaling pathways in the tumor cell. The alternative mechanism for PD-L1 upregulation in tumors is their adaptation to endogenous tumor-specific immune responses to IFNs, predominantly IFN- γ , in a process termed adaptive immune resistance [42]. Regarding lung SCC samples in our study, PD-L1 expression significantly correlated with PTEN expression. Eighty percent of PD-L1-negative cases showed PTEN loss ($P = 0.005$). Thus, upregulation of PD-L1 in these PD-L1-positive tumor cells may occur via adaptive immune resistance. It may be assumed that PTEN-positive tumor cells evade antitumor immunity by upregulating the expression of PD-L1. It may also be deduced that lung SCC characterized by the loss of the tumor suppressor PTEN can proceed to tumorigenesis without PD-L1 expression.

Additionally, we found that smoking significantly correlated with PTEN loss in ADC. It is possible that smoking influences the expression of *PTEN* gene to a certain extent. Indeed; one study concluded that cigarette smoke induced alterations in the PI3K/PTEN balance in human airway epithelial cells [43].

A limitation of our study is that we evaluated only PTEN expression but not that of other molecular oncogene markers of PI3K and downstream pathways involved in tumor formation. Furthermore, we did not examine factors influencing PD-L1 expression in tumor cells. In addition, we did not study tumor-infiltrating cells, believed to control the response to anti-PD-L1 therapy. Although we used whole tissue sections for ASC, we had only 28 samples, given the rarity of the tumor. Although we sought to retrieve as much ASC clinicopathological data as possible, some data were missing from the electronic records of old cases.

In conclusion, our study provides an insight into the biological mechanisms underlying the immune and oncogenic nature of adenocarcinoma cell carcinoma. Further multi-institutional studies are required to reproduce these findings and establish their biological and clinical importance.

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Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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