Int J Clin Exp Pathol 2017;10(6):6935-6945
www.ijcep.com /ISSN:1936-2625/IJCEP0049992

Original Article

Differential contributions of adeno- and squamous components in promoting lymphatic metastasis of lung adenosquamous cell carcinoma

Haoshuai Zhu[1,2*], Jianyong Zou[1,2*], Chunhua Su[1,2*], Yiyan Lei[1,2], Bo Zeng[1,2], Shuisheng Zhang[1,2], Honghe Luo[1,2], Zhenguang Chen[1,2,3]

Departments of [1]Thoracic Surgery, [3]Cardiothoracic Surgery of East Division, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China; [2]Lung Cancer Research Center of Sun Yat-sen University, Guangzhou, Guangdong, China. [*]Equal contributors.

Received February 3, 2017; Accepted April 17, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: Background: Pulmonary adenosquamous carcinoma (ASC) is a rare type of lung cancer containing both adenocarcinoma (AC) and squamous cell carcinoma (SCC) cells. To better understand the mechanism by which the AC and SCC components are involved in lymph node metastasis, we evaluated the distribution of these cellular components in both primary ASC tumors and lymph node metastases. Methods: Twenty-two patients with ASC and 142 with AC were admitted to the First Affiliated Hospital of Sun Yat-sen University between July 2006 and July 2012 and underwent surgical resection. Primary ASCs and lymph nodes, with or without metastasis, were assayed immunohistochemically for TTF-1, p63, p40, and VEGF-C. Results: The incidence of lymph node metastasis was higher in patients with ASCs than ACs; however, other features of ASC and AC showed no differences. ASC tumors were positive for TTF-1, p63, and p40; however, these proteins were distributed in different tumor islands in the same tumor. Co-expression was less frequent, but TTF-1 expression was higher in metastatic lymph nodes. Conclusions: ASCs are highly aggressive tumors that metastasize via the lymphatic pathway. The two cellular components of these tumors (AC and SCC) behave independently, with the AC component playing a predominant role in lymphatic invasion. Metastatic lymph nodes should be evaluated immunohistochemically to choose a proper chemotherapeutic strategy in patients with ASCs.

Keywords: Lung cancer, pulmonary adenosquamous carcinoma, lymph node, metastasis

Introduction

Adenosquamous carcinoma (ASC) of the lung, defined as a mixture of adenocarcinoma (AC) and squamous cell carcinoma (SCC), is a rare subtype of non-small cell lung cancer (NSCLC) that is present in 0.5-5% of patients with NSCLC [1]. Because lung carcinoma is inherently heterogeneous, there remains persistent uncertainty regarding the histogenesis, clinical, and histopathologic features of ASC [2]. In one study that included 1,284 patients with primary lung cancer, 44 (3.4%) patients had ASCs and had a cumulative 5-year postoperative survival rate of only 18.5%, markedly lower than in patients with SCCs and ACs [3]. Another study, in which 141 of 5,218 (2.7%) patients with primary lung cancer had ASCs, found that ASCs were associated with larger tumor size and more frequent visceral pleura invasion, microinvasion of the lymphatic vessels, and ipsilateral second nodules compared to ACs and SCCs [4]. Among the patients with documented ASCs, 48% presented with a combination of AC and SCC tumor cells, with each component contributing 40-60% of the tumor, and 55% were associated with undifferentiated large cells. Interestingly, although ASCs were associated with a lower 5-year survival rate (37%) than ACs (42.8%) and SCCs (43.4%), survival has improved over the last decade, especially in patients with balanced AC and SCC components [5]. ASCs are highly aggressive and frequently accompanied by lymph node metastasis; consequently, adjuvant chemotherapy is recommended even in patients with early stage

Table 1. Clinicopathological features of ASC and AC

	ASC	AC	*P*-values
Number of Patients	22	142	
Age, years, Median (Range)	57 (30-70)	59 (32-79)	0.56[b]
Sex			
Male	14 (77.3%)	83 (58.5%)	0.65[a]
Female	8 (22.7%)	59 (41.5%)	
Smoking Status			
Former/current smoker	7 (32%)	84 (59%)	0.03[a]
Never smoker	15 (68%)	58 (41%)	
Tumor differentiation			
Well	3 (13.6%)	20 (14.1%)	1.00[c]
Moderate	11 (50%)	70 (49.3%)	
Poor	8 (36.4%)	52 (36.6%)	
Lymph nodes involvement			
N_0	4 (18.2%)	80 (56.3%)	0.001[c]
N_1	13 (59.0%)	27 (19%)	
N_2	5 (22.8%)	35 (24.6%)	
EGFR mutations			
Positive	5 (22.7%)	49 (34.5%)	0.27[a]
	21 exon 3 (13.6%)	21 exon 34 (23.9%)	
	19 exon 2 (9.1%)	19 exon 15 (10.6%)	
Negative	17 (77.3%)	93 (65.5%)	
Kras mutations			
Positive	1 (4.5%)	12 (8.5%)	0.53[a]
Negative	21 (95.5%)	130 (91.5%)	
UICC stages			
I	4 (18.2%)	65 (45.8%)	0.07[a]
II	12 (54.5%)	34 (23.9%)	
III	6 (27.3%)	43 (30.3%)	
IV	0	0	

Abbreviations: ASC, adenosquamous carcinoma; AC, asenocarcinoma; EGFR, epidermal growth factor receptor; Kras, Kirsten rat sarcoma viral oncogene homolog; UICC, the Union Internationale Contre le Cancer; a. By Chi-square test; b. By Anova test. c. By Fisher exact's test.

disease [6, 7]. However, the mechanisms underlying this aggressive tumor behavior remain unclear, raising questions about optimal treatment [8]. Several recent studies have suggested that ASCs are not simply mixtures of AC and SCC cells, but that the two components behave as a single entity [9-11]. Four biomarkers have been analyzed to better understand the mechanisms by which the AC and SCC components are involved in lymph node metastasis: (1) thyroid transcription factor-1 (TTF-1), a specific marker for lung ACs, (2) transformation-related protein 63 (p63) and (3) an isoform of the p63 protein without the transactivation domain (p40), which are both specific markers for SCCs, and (4) vascular endothelial growth factor C (VEGFC), a specific marker for lymphangiogenesis [8-12].

TTF-1, also known as NK-X2-1, is a homeobox-containing transcription factor that is essential for lung, thyroid, and brain development. During the pathogenesis of lung AC, TTF-1 plays a role as a lineage-survival oncogene and is associated with the inhibition of tumor invasion and metastasis. TTF-1 is expressed in 70% of lung ACs and is expressed in all disease stages, but is rarely expressed in lung SCC [13, 14]. p63 is a homolog of p53 that regulates programmed cell death, development, and differentiation. Although the role of p63 in the development of lung cancer is unclear, it has been shown to be a marker of lung SCC [15-17]. Thus, co-expression of TTF-1 and p63 in cancer cells is considered a specific pathological characteristic of primary ASCs. However, the expression of these markers in ASC lymph node metastases has not been determined [17, 18]. VEGF-C is a member of the VEGF family and is involved in angiogenesis and lymphangiogenesis to promote cancer metastasis [19, 20]. Using antibodies to these proteins, we analyzed lymph nodes from patients with ASC to determine the contribution of AC- and SCC-derived cells to metastasis. The poor prognosis and aggressive nature of ASCs have led to the recommendation that adjuvant chemotherapy be administered to all patients with these tumors, even those with stage I disease [21]. However, the optimal chemotherapy regimen has not been determined. Expression of thymidylate synthase, the main target of pemetrexed, has been observed in ASCs, suggesting that

Table 2. Multivariate logistic regression analysis of the risk factors associated with ASC (Sample size 164)

Variables	ASC (%) (N=22)	AC (%) (N=142)	OR	95% confidence interval	*P*-value
Age (years)					
<60 years old	10 (45.5%)	60 (42.9%)	0.83	0.31-2.19	0.7
≥60 years old	12 (54.5%)	80 (57.1%)			
Gender					
Male	14 (77.3%)	83 (58.5%)	2.78	0.53-14.72	0.23
Female	8 (22.7%)	59 (41.5%)			
Smoking Status					
Former/current smoker	7 (32%)	84 (59%)	0.69	0.14-3.33	0.64
Never smoker	15 (68%)	58 (41%)			
Tumor differentiation					
Well	3 (13.6%)	20 (14.1%)	1.39	0.34-5.70	0.65
Moderate + poor	19 (86.4%)	122 (85.9%)			
Lymph nodes involvement					
Yes	17 (77.3%)	62 (43.7%)	9.41	1.72-51.48	0.01
No	5 (22.7%)	80 (56.3%)			
EGFR mutations					
Positive	5 (22.7%)	49 (34.5%)	1.46	0.47-4.55	0.52
Negative	17 (77.3%)	93 (65.5%)			
Kras mutations					
Positive	1 (4.5%)	12 (8.5%)	1.84	0.21-16.45	0.59
Negative	21 (95.5%)	130 (91.5%)			
UICC stages					
I	4 (18.2%)	65 (45.8%)	1.92	0.73-5.10	0.19
II + III	18 (81.8%)	77 (54.2%)			

Abbreviations: ASC, adenosquamous carcinoma; AC, asenocarcinoma; EGFR, epidermal growth factor receptor; Kras, Kirsten rat sarcoma viral oncogene homolog; UICC, the Union Internationale Contre le Cancer.

this agent may be efficacious in treating patients with ASCs [8]. In contrast, SCCs respond poorly to pemetrexed, suggesting that ASCs may not respond to this agent [22]. This contradiction requires further exploration to determine whether ASCs respond to pemetrexed-based regimens.

In the present study, we aimed to evaluate the distribution of AC and SCC cellular components in primary ASC tumors and lymph nodes, and to determine the biological involvement of these components in lymph node metastasis.

Materials and methods

Patients

Twenty-two patients with ASC, defined according to the 2004 World Health Organization (WHO) classification of lung carcinoma, were admitted between July 2006 and July 2012 to the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, and underwent standard radical lobectomies [23]. The pathological diagnosis of ASC was confirmed by at least two experienced pathologists based on cellular morphology according to the WHO classification of lung carcinoma and immunochemistry staining, including TTF-1, CK7, CK, p63, and M-CEA expression. Lymph nodes metastases were observed in 18 patients. During the same time period, samples from 142 patients who underwent surgical resection for AC were collected. Tumors and regional lymph node tissue samples were obtained from the pathology department, and clinical data were summarized. Disease stage was determined using the Tumor, Node, and Metastasis (TNM) and Union Internationale Contrele Cancer (UICC) staging systems [24]. The Medical Ethical Committee of The First Affiliated Hospital, Sun Yat-sen University approved the use of human materials. (Full name of the board committee: the Medical Ethical Committee of The First Affiliated Hospital, Sun Yat-sen University. No. 2009-11). All research involving humans was performed in compliance with the Declaration of Helsinki. Written informed consent for participation in the study was obtained from all participants.

Immunohistochemical staining

H&E and immunohistochemical staining using antibodies against TTF-1, p63, p40, M-CEA, and CK (Abcam, Cambridge, UK) were performed to confirm the diagnosis of ASC or AC. A

Table 3. Expression of TTF-1 and p63 in 22 patients with ASC

	Primary Tumor	Lymph nodes	*P*-Values
TTF-1 expression			
Positive	17 (77.3%)	12 (54.5%)	0.112[a]
Negative	5 (22.7%)	10 (45.5%)	
P63 expression			
Positive	18 (81.8%)	9 (40.9%)	0.012[b]
Negative	4 (18.2%)	13 (59.1%)	
P40 expression			
Positive	19 (86.4%)	10 (45.5%)	0.01[b]
Negative	3 (13.6%)	12 (54.5%)	
TTF-1^{positive} P63^{positive}	16 (72.7%)	5 (22.7%)	0.001[b]
TTF-1^{positive} P63^{negative}	1 (4.5%)	7 (31.8%)	
TTF-1^{negative} P63^{positive}	2 (9.1%)	4 (18.2%)	
TTF-1^{negative} P63^{negative}	3 (13.7%)	6 (27.3%)	

Abbreviations: ASC, adenosquamous carcinoma; TTF-1, thyroid transcription factor-1; p63, transformation-related protein 63; P40, a truncated isoform of p63. a. By Chi-square test. b. By Fisher exact's test.

Table 4. Expression of TTF-1 and P63 in 18 patients with ASC and lymph node metastasis

	Primary Tumor	Lymph nodes	*P*-Values
TTF-1 expression			
Positive	15 (83.3%)	12 (66.7%)	0.22[a]
Negative	3 (16.7%)	6 (33.3%)	
P63 expression			
Positive	16 (88.9%)	9 (50%)	0.03[a]
Negative	2 (11.1%)	9 (50%)	
P40 expression			
Positive	16 (88.9%)	10 (55.6%)	0.06[a]
Negative	2 (11.1%)	8 (44.4%)	
TTF-1^{positive} P63^{positive}	14 (77.8%)	5 (27.8%)	0.012[a]
TTF-1^{positive} P63^{negative}	1 (5.5%)	7 (38.9%)	
TTF-1^{negative} P63^{positive}	2 (11.2%)	4 (22.2%)	
TTF-1^{negative} P63^{negative}	1 (5.5%)	2 (11.1%)	

Abbreviations: ASC, adenosquamous carcinoma; TTF-1, thyroid transcription factor-1; p63, transformation-related protein 63. P40, a truncated isoform of p63. a. By Fisher exact's test.

diagnosis of ASC was made if SCC and AC components each accounted for >10% of the tumor. Using the same antibodies, TTF-1, p63, p40, and VEFG-C were assayed in tissue samples taken from the lymph nodes of the 18 ASC patients with positive metastasis and the four ASC patients with node-negative disease.

Tissue sections were deparaffinized with xylene, rehydrated with ethanol, and incubated with 3% hydrogen peroxide to block endogenous peroxidase activity [7]. Following antigen retrieval, the slides were incubated overnight at 4°C with the above-described primary antibodies. Following incubation with secondary antibodies, binding was detected using the labeled streptavidin biotin (LSAB) method according to the manufacturer's instructions (Dako Cytomation, Carpinteria, CA, USA). Samples showing nuclear staining for TTF-1, p63, and p40 in >10% of tumor cells were considered positive for that respective component. Cytoplasm staining for VEGF-C was conducted to evaluate lymphatic vessel density and lymphangiogenesis based on the ratio and intensity of positive-staining cells: 0-10%, scored 0; 11-30%, scored 1; 31-60%, scored 2; 61-100%, scored 3 [25].

Statistical analysis

All calculations were performed using SPSS statistical software v19.0 (Chicago, IL, USA). Comparison of ratios was done by Chi-square test with correction coefficient or Fisher's exact test if necessary. Multivariate analyses were used to evaluate risk factors by Logistic regression model. Analysis of variance (ANOVA) followed by LSD were used to campare means across groups. A p-value <0.05 was considered statistically significant.

Results

Patient characteristics

The clinicopathological characteristics of the 22 patients with ASCs and the 142 with ACs are summarized in **Table 1**. The median ages (ASC: 57 [range, 30-70 years] vs. AC: 59 [range, 32-79 years], P=0.56) and sex distribution (P=0.65) were similar, with both groups showing male predominance. Patients with ASCs smoked less heavily than those with ACs (P=0.03). Tumor differentiation was similar. The frequency of K-ras mutation was similar (P=0.53). EGFR mutations, which included 19 exon deletions and L858R, were not different between the two groups (P=0.27). UICC stage tended to be higher in patients with ASCs than ACs (P=0.07). Lymph node metastasis occurred significantly more frequently in patients with ASCs than ACs (77.3% vs. 43.7%,

Table 5. Clinicopathological features of 18 ASC with or without TTF-1 expression in metastasized lymph node

	TTF-1 expression positive	TTF-1 expression negative	*P*-values
Number of Patients	12	6	
Age, years, Median (Range)	59 (30-65)	55 (44-71)	0.33[b]
Sex			
Male	6 (50%)	4 (66.7%)	0.64[a]
Female	6 (50%)	2 (33.3%)	
Smoking Status			
Former/current smoker	4 (33.3%)	2 (33.3%)	1.00[a]
Never smoker	8 (66.7%)	4 (66.7%)	
Tumor differentiation			
Well	1 (8.3%)	1 (16.7%)	1.00[a]
Moderate	6 (50%)	3 (49.3%)	
Poor	5 (41.7%)	2 (33.3%)	
EGFR mutations			
Positive	2 (16.7%)	2 (33.3%)	0.57[a]
Negative	10 (83.3%)	4 (66.7%)	
Kras mutations			
Positive	1 (8.3%)	0 (0%)	1.00[a]
Negative	11 (91.7%)	6 (100%)	
UICC stages			
I	0 (0%)	0 (0%)	0.62[a]
II	8 (66.7%)	5 (83.3%)	
III	4 (33.3%)	1 (16.7%)	
IV	0 (0%)	0 (0%)	
TTF-1 expression in tumor			
Positive	12 (100%)	3 (50%)	0.03[a]
Negative	0 (0%)	3 (50%)	
P63 expression in tumor			
Positive	12 (100%)	4 (66.7%)	0.09[a]
Negative	0 (0%)	2 (33.3%)	
P63 expression in lymph nodes			
Positive	7 (58.3%)	4 (66.7%)	1.00[a]
Negative	5 (41.7%)	2 (33.3%)	
VEGF-C expression in tumor (score)			
0-1	6 (50%)	5 (83.3%)	0.32[a]
3-4	6 (50%)	1 (16.7%)	
VEGF-C expression in lymph nodes (score)			
0-1	5 (41.7%)	5 (83.3%)	0.15[a]
3-4	7 (58.3%)	1 (16.7%)	

Abbreviations: ASC, adenosquamous carcinoma; AC, asenocarcinoma; EGFR, epidermal growth factor receptor; Kras, Kirsten rat sarcoma viral oncogene homolog; UICC, the Union Internationale Contre le Cancer; TTF-1, thyroid transcription factor-1; p63, transformation-related protein 63; VEGF-C, vascular endothelial growth factor-C; a. By Fisher exact's test; b. By Anova test.

P=0.001). Multivariate logistic regression analysis of the risk factors associated with ASC was performed; the data are shown in **Table 2**. Through this analysis, lymph node involvement was strongly associated with ASCs.

Immunohistochemical analysis

TTF-1 was positive in 17 of the 22 primary ASCs (77.3%) and in lymph nodes from 12 (54.5%) of the 22 patients (*P*=0.112). p63 expression was positive in 18 (81.8%) primary ASC tumors and in 9 (40.9%) lymph nodes (*P*=0.012). p40 expression was positive in 19 (86.4%) primary ASC tumors and in the lymph nodes of 10 (45.3%) patients (*P*=0.01). The expression of p63 and p40 were unanimous in the present study. For further analysis, patients with ASCs were divided into four groups: TTF-$1^{positive}$ $63^{positive}$, TTF-$1^{positive}$ $63^{negative}$, TTF-$1^{negative}$ $63^{positive}$, and TTF-$1^{negative}$ $63^{negative}$. In an analysis of the primary tumors, 16 tumors were positive for both markers, 1 was positive for TTF-1 alone, 2 were positive for p63 alone, and 3 were negative for both markers. In an analysis of lymph nodes, 5 cases were positive for TTF-1 and p63, 7 were positive for TTF-1 alone, and 4 were positive for p63 alone. The lymph nodes from six patients, including all 4 patients with node-negative (N_0) disease, were negative for both tumor markers. In general, the expression of TTF-1 and p63 differed significantly between the primary tumors and lymph nodes (*P*=0.001; **Table 3**). To better understand lymph node metastasis in ASC, the four patients with

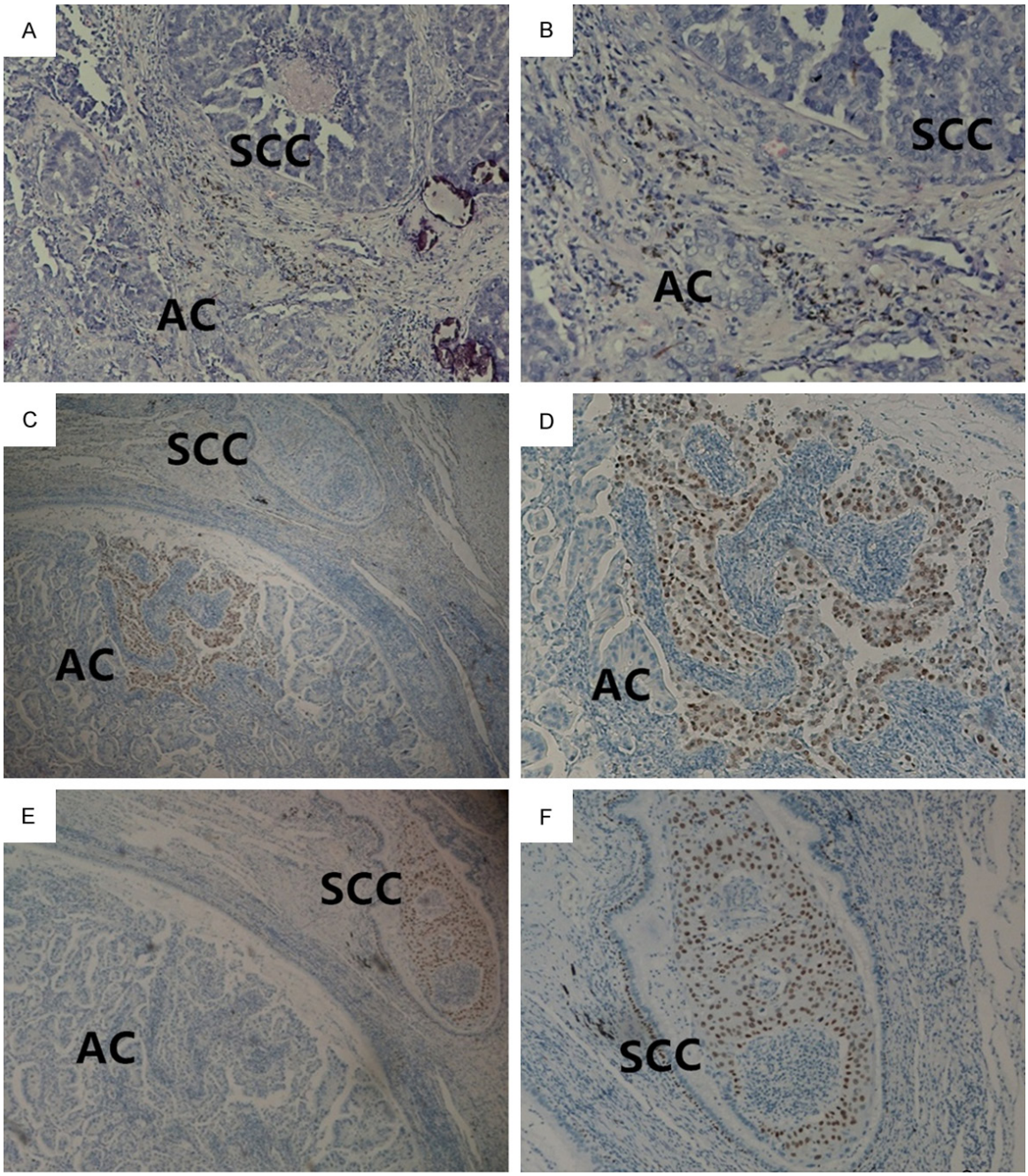

Figure 1. Components of adenocarcinoma (AC) and squamous cell carcinoma (SCC) in tumor sites. (A, B) HE staining, showing that components of AC and SCC are present in different positions of the tumor rather than mixing together. (C, D) Staining for TTF-1, showing that TTF-1 is located in the nuclei of adenocarcinoma cells. (E, F) Staining for p63, showing that p63 is located in the nuclei of squamous cell carcinoma cells. The separation of TTF-1 from p63 in the same visual fields confirmed the HE findings (A, C, E, 10×; B, D, F, 20×).

N_0 disease were excluded, and the distribution of AC and SCC components at both the primary tumor site and in the lymph nodes were assayed in the remaining 18 patients with nodal involvement (**Table 4**). Of the 18 primary tumors, 15 were positive for TTF-1 expression, 16 for p63 expression, and 16 for p40 expression (P=0.22). Assays of lymph node tissues from these patients showed that 12 were positive for TTF-1, 9 for p63, and 10 for p40. The expression of p63 differed significantly between primary tumors and lymph nodes (P=0.003), while the expression of TTF-1 was similar (P=0.22). Fourteen primary tumors and 5 lymph nodes

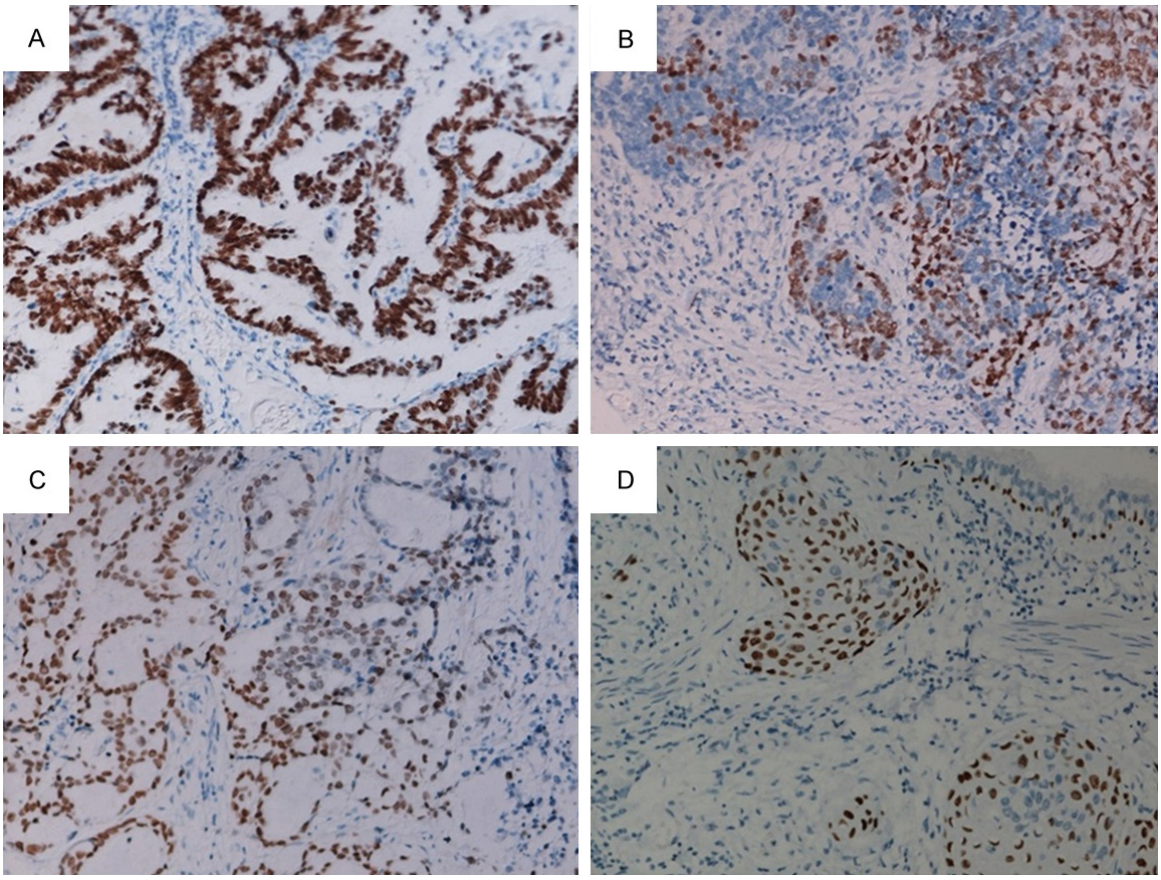

Figure 2. Presence of TTF-1 and p63 in metastatic lymph nodes and at tumor sites. (A, B) TTF-1 and p63 were more abundant at primary tumor sites than in metastatic lymph nodes sites. (C, D) In metastatic lymph nodes, the limited distributions of AC and SCC cells surrounded by lymph cells were identified by the presence of TTF-1 and p63, respectively (A-D, 20×).

were positive for both TTF-1 and p63 (P=0.012). The characteristics of the 18 ASC patients with lymph node involvement were compared with regards to TTF-1 expression in metastatic lymph nodes (**Table 5**). The expressions of TTF-1 and p63 in primary tumors were significantly higher in the patients with positive TTF-1 expression in metastatic lymph nodes compared with those with TTF-1^{negative} nodes (P=0.003). The expression of p63 in both primary tumors and lymph node sites were not significantly different (P=0.09, P=1.00, respectively). VEGF-C expression scores in primary tumors and in lymph nodes were not different between the cases with and without TTF-1 expression in the metastatic lymph nodes (P=0.32, P=0.15, respectively).

Discussion

Aggressive tumor progression and poor prognosis are clinical characteristics of ASC [26]. However, the mechanisms that promote invasiveness in ASC have not been determined. Patients with ASC have poorer prognoses than those with AC, even when tumor stage and lymph node involvement are similar [5, 9]. While the incidence of lymph-node metastasis has been shown to be significantly higher in patients with ASC than in patient with other types of lung cancers [26, 27], lymph-node involvement is still an important characteristic of lung AC. Therefore, to assess the differential contributions of AC and SCC components in promoting lymphatic metastasis in ASC, we compared cell components from ASC to AC. We found that although tumor stages were similar in patients with ASC and AC, lymph node metastasis was seen more frequently in patients with ASC, suggesting that ASC invasion prefers a lymphatic approach. ASC, however, is not simply a mixture of the two histological components (AC and SCC); ASC behaves as a single entity [7], a find-

ing supported by the present results. H&E and immunohistochemical analyses showed that the glandular structures of AC and carcinoma nests of SCC were separate, as seen by separation of malignant cells that were positive for TTF-1 and p63 in ASC tumors rather than being mixed together (**Figure 1**). This separation may provide a relatively independent microenvironment for each of these components. AC and SCC components in lymph node metastases were labeled with antibodies to TTF-1 and p63, respectively. TTF-1 and p63 are considered the most reliable markers to distinguish AC and SCC in the lungs [28]. TTF-1 is one of the master regulatory genes in lung development and is a lineage marker of TRU in lung carcinogenesis. p63 is a nuclear protein homologous to p53 that is expressed in basal cells of stratified squamous and glandular epithelia. Of the 22 primary ASCs, 17 (77.3%) were positive for TTF-1 and 18 (81.8%) for p63, with 16 (72.3%) tumors being positive for both. Interestingly, only 9 ASC patients (40.0%) had lymph nodes that were positive for p63, whereas 12 patients (54.5%) were positive for TTF-1. Of the 18 patients positive for lymph node metastasis, fewer patients had lymph node metastases positive for p63 than the number of patients with positive primary tumors; contrarily, the number of patients positive for TTF-1 did not differ significantly by site. Cancer cells that invade lymph nodes show changes in structure. ASC is a special type of cancer containing two different components [29]. When these cells invade adjacent lymph nodes, the components are difficult to distinguish by their morphological features. To our knowledge, this study is the first to use antibodies to TTF-1, p63, and p40 to identify ASC components in metastatic lymph nodes. We found that a higher percentage of lymph nodes were positive for TTF-1 than for p63, suggesting that AC components of ASC may occur more frequently in metastatic lymph nodes than the SCC component. The AC component may play a predominant role in tumor metastasis through the lymphatic system (**Figure 2**). We also found that fewer patients had lymph nodes that were TTF-1- and p63-positive than primary tumors, with lymph nodes being more frequently positive for single markers, suggesting that the two components of ASC invade lymph nodes independently. Additionally, metastatic lymph nodes that were positive for TTF-1 tended to have higher VEGF-C expression; however, small sample size prevented meaningful statistical analysis. Since ASC is an aggressive malignancy that carries a poor patient prognosis, adjuvant chemotherapy is recommended, even in patients with stage I tumors [5]. In ASC cells, expression of thymidylate synthase, the main target of pemetrexed, was discovered, suggesting that this agent could be used to treat ASC [7, 8]. However, SCC has shown to have a poor response to premetrexed [18, 19], suggesting that some cells in ASC may not respond to this agent. A study in patients with laryngeal adenosquamous carcinoma suggested that metastatic lymph nodes should be evaluated due to the nature of adenosquamous carcinomas to spread, and that the results of the lymph node involvement could guide further treatment [30]. We found that metastatic lymph nodes were more frequently positive for TTF-1 than p63, suggesting that the AC component of ASC may contribute more to lymph node metastasis. Alternatively, if AC and SCC components contribute equally to lymph node metastasis, the heterogeneity of SCC may result in the down regulation of p63 expression in these cells after they migrate into the lymph nodes. If the first hypothesis is true, pemetrexed may be effective in treating patients with ASCs, especially those with N_0 disease. Prospective studies and additional studies with more precise methods of differentiating the ACC and SCC components in ASC are required to test this hypothesis.

Since epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) may be effective in some histologic types of NSCLC, patients with ASCs should be assayed for EGFR mutations, especially those with disease recurrence [31]. Drug response-specific gene alterations may occur in both the AC and SCC components of ASC, suggesting that patients with confirmed or suspected ASCs should undergo driver gene analyses [32, 33].

The present study had two major limitations. The first was the small size of the ASC group, due mainly to the rarity of this disease. Multicenter studies may be needed in the future to enroll a larger sample of patients. Second, this study was retrospective in design, suggesting the need for additional prospective studies. Moreover, the inherent heterogeneity of ASCs gives rise to uncertainties regarding the histogenesis and clinical and histopathologic features of the disease [2].

In conclusion, our results suggest that ASC metastasizes primarily through lymphatic pathways. Invasion by the AC component is more common than metastasis by the SCC component or mixed metastasis of both components. These findings reveal the biological behaviors of ASC in promoting lymphatic metastasis and may give physicians some guidance upon encountering this rare type of lung cancer. Our results suggest that pemetrexed may be an appropriate adjuvant chemotherapy agent for patients with ASC without lymphatic metastasis. However, further analysis in patients with nodal involvement (N_1 or N_2 disease) is required to determine which component is more prominent in lymphatic metastasis before adjuvant therapy. Staining of primary tumors and metastatic lymph nodes showed that TTF-1 was more frequently expressed than p63 in metastatic lymph nodes, and that the expression of these two markers was distributed in different sites in the same lymph node section. The AC component of ASC may play a greater role than SCC in lymph node metastasis, with the two components likely behaving independently.

Acknowledgements

All procedures performed in studies involving human participants were in accordance with the ethical standards of the 1st affiliated hospital of Sun Yat-sen university research committee and with the 1964 Helsinki declaration and its later amendments.

Informed consent was obtained from all individual participants included in the study.

Disclosure of conflict of interest

None.

Address correspondence to: Zhenguang Chen, Department of Thoracic Surgery, Cardiothoracic Surgery of East Division, The First Affiliated Hospital, Sun Yat-sen University, Lung Cancer Research Center of Sun Yat-sen University, 58 Zhongshan Road II, Guangzhou 510080, Guangdong, China. E-mail: chenzhenguang@yahoo.com

References

[1] Shimizu J, Oda M, Hayashi Y, Nonomura A, Watanabe Y. A clinicopathologic study of resected cases of adenosquamous carcinoma of the lung. Chest 1996; 109: 989-994.

[2] Rao N. Adenosquamous carcinoma. Semin Diagn Pathol 2014; 31: 271-277.

[3] Shundo Y, Takahashi T, Itaya T, Neyatani H, Sugimura H, Kita Y, Nogimura H, Kobayashi R, Suzuki K, Shimizu K, Funai K, Shiiya N. Clinical study of forty-two patients who underwent resection for pulmonary adenosquamous carcinoma. Kyobu Geka 2011; 64: 871-876; discussion 876-879.

[4] Li B, Li Z, Zhang Y, Li Y, Wu W. Clinical analysis of surgically treated patients with adenosquamous carcinoma of the lung. Zhongguo Fei Ai Za Zhi 2006; 9: 469-470.

[5] Mordant P, Grand B, Cazes A, Foucault C, Dujon A, Le Pimpec Barthes F, Riquet M. Adenosquamous carcinoma of the lung: surgical management, pathologic characteristics, and prognostic implications. Ann Thorac Surg 2013; 95: 1189-1195.

[6] Maeda H, matsumura A, Kawabata T, Suito T, Kawashima O, Watanabe T, Okabayashi K, Kubota I; Japan National Hospital Organization Study Group for Lung Cancer. Adenosquamous carcinoma of the lung: surgical results as compared with squamous cell and adenocarcinoma cases. Eur J Cardiothorac Surg 2012; 41: 357-361.

[7] Filosso PL, Ruffini E, Asioli S, Giobbe R, Macri L, Bruna MC, Sandri A, Oliaro A. Adenosquamous lung carcinomas: a histologic subtype with poor prognosis. Lung Cancer 2011; 74: 25-29.

[8] Shu C, Cheng H, Wang A, Mansukhani MM, Powell CA, Halmos B, Borczuk AC. Thymidylate synthase expression and molecular alterations in adenosquamous carcinoma of the lung. Mod Pathol 2013; 26: 239-246.

[9] Wang R, Pan Y, Li C, Zhang H, Garfield D, Li Y, Ye T, Hu H, Luo X, Li H, Zhang Y, Zhang J, Zhou X, Shen L, Pao W, Sun Y, Chen H. Analysis of major known driver mutations and prognosis in resected adenosquamous lung carcinomas. J Thorac Oncol 2014; 9: 760-768.

[10] Dragnev KH, Gehr G, Memoli VA, Tafe LJ. ALK-rearranged adenosquamous lung cancer presenting as squamous cell carcinoma: a potential challenge to histologic type triaging of NSCLC biopsies for molecular studies. Clin Lung Cancer 2014; 15: e37-40.

[11] Powrózek T, Krawczyk P, Ramlau R, Sura S, Wojas-Krawczyk K, K ucharczyk T, Walczyna B, Szumiło J, Szyszka-Barth K, Milecki P, Barinow-Wojewódzki A, Milanowski J. EGFR gene mutations in patients with adenosquamous lung carcinoma. Asia Pac J Clin Oncol 2014; 10: 340-345.

[12] Shimoji M, Nakajima T, Yamatani C, Yamamoto M, Saishou S, Isaka M, Maniwa T, Ode Y, Nakagawa K, Okumura T, Watanabe R, Ito I, Kameya T, Endo M, Kondo H. A clinicopatho-

logical and immunohistological re-evaluation of adenosquamous carcinoma of the lung. Pathol Int 2011; 61: 717-722.
[13] Yamaguchi T, Hosono Y, Yanagisawa K, Takahashi T. NKX2-1/TTF-1: an enigmatic oncogene that functions as a double-edged sword for cancer cell survival and progression. Cancer Cell 2013; 23: 718-723.
[14] Rossi G, Pelosi G, Barbareschi M, Graziano P, Cavazza A, Papotti M. Subtyping non-small cell lung cancer: relevant issues and operative recommendations for the best pathology practice. Int J Surg Pathol 2013; 21: 326-336.
[15] Conde E, Angulo B, Redondo P, Toldos O, García-García E, Suárez-Gauthier A, Rubio-Viqueira B, Marrón C, García-Luján R, Sánchez-Céspedes M, López-Encuentra A, Paz-Ares L, López-Ríos F. The use of P63 immunohistochemistry for the identification of squamous cell carcinoma of the lung. PLoS One 2010; 5: e12209.
[16] Yao JY, Chen JK. Roles of p63 in epidermal development and tumorigenesis. Biomed J 2012; 35: 457-463.
[17] Su X, Chakravarti D, Flores ER. p63 steps into the limelight: crucial roles in the suppression of tumorigenesis and metastasis. Nat Rev Cancer 2013; 13: 136-143.
[18] Brunnstrom H, Johansson L, Jirström K, Jönsson M, Jönsson P, Planck M. Immunohistochemistry in the differential diagnostics of primary lung cancer: an investigation within the Southern Swedish lung cancer study. Am J Clin Pathol 2013; 140: 37-46.
[19] Zhang Y, Meng X, Zeng H, Guan Y, Zhang Q, Guo S, Liu X, Guo Q. Serum vascular endothelial growth factor-C levels: a possible diagnostic marker for lymph node metastasis in patients with primary non-small cell lung cancer. Oncol Lett 2013; 6: 545-549.
[20] Chen Z, Wang T, Luo H, Lai Y, Yang X, Li F, Lei Y, Su C, Zhang X, Lahn BT, Xiang AP. Expression of nestin in lymph node metastasis and lymphangiogenesis in non-small cell lung cancer patients. Hum Pathol 2010; 41: 737-744.
[21] Bastide K, Ugolin N, Levalois C, Bernaudin JF, Chevillard S. Are adenosquamous lung carcinomas a simple mix of adenocarcinomas and squamous cell carcinomas, or more complex at the molecular level? Lung Cancer 2010; 68: 1-9.
[22] Paz-Ares L, de Marinis F, Dediu M, Thomas M, Pujol JL, Bidoli P, Molinier O, Sahoo TP, Laack E, Reck M, Corral J, Melemed S, John W, Chouaki N, Zimmermann AH, Visseren-Grul C, Gridelli C. Maintenance therapy with pemetrexed plus best supportive care versus placebo plus best supportive care after induction therapy with pemetrexed plus cisplatin for advanced non-squamous non-small-cell lung cancer (PARAMOUNT): a double-blind, phase 3, randomised controlled trial. Lancet Oncol 2012; 13: 247-255.
[23] World Health Organization classification of tumours. Pathology and genetics. In: tumours of the lung, pleura, thymus and heart. Lyon: IARC Press; 2004.
[24] Tsim S, O'Dowd CA, Milroy R, Davidson S. Staging of non-small cell lung cancer (NSCLC): a review. Respir Med 2010; 104: 1767-1774.
[25] Song WW, Lu H, Hou WJ, Xu GX, Zhang JH, Sheng YH, Cheng MJ, Zhang R. Expression of vascular endothelial growth factor C and anti-angiogenesis therapy in endometriosis. Int J Clin Exp Pathol 2014; 7: 7752-7759.
[26] Uramoto H, Yamada S, Hanagiri T. Clinicopathological characteristics of resected adenosquamous cell carcinoma of the lung: risk of coexistent double cancer. J Cardiothorac Surg 2010; 5: 92.
[27] Naunheim KS, Taylor JR, Skosey C, Hoffman PC, Ferguson MK, Golomb HM, Little AG. Adenosquamous lung carcinoma: clinical characteristics, treatment, and prognosis. Ann Thorac Surg 1987; 44: 462-466.
[28] Yamatani C, Abe M, Shimoji M, Maniwa T, Takahashi S, Isaka M, Ohde Y, Watanabe R, Ito I, Kondo H, Nakajima T. Pulmonary adenosquamous carcinoma with mucoepidermoid carcinoma-like component with characteristic p63 staining pattern: either a novel subtype originating from bronchial epithelium or variant mucoepidermoid carcinoma. Lung Cancer 2014; 84: 45-50.
[29] Shimoji M, Nakajima T, Yamatani C, Yamamoto M, Saishou S, Isaka M, Maniwa T, Ode Y, Nakagawa K, Okumura T, Watanabe R, Ito I, Kameya T, Endo M, Kondo H. A clinicopathological and immunohistological re-evaluation of adenosquamous carcinoma of the lung. Pathol Int 2011; 61: 717-722.
[30] Mokhtari S and Mohsenifar Z. Diagnostic histological features of metastatic lymph nodes in adenosquamous carcinoma. Pathol Res Pract 2012; 208: 360-362.
[31] Jia XL, Chen G. EGFR and KRAS mutations in Chinese patients with adenosquamous carcinoma of the lung. Lung Cancer 2011; 74: 396-400.
[32] Morodomi Y, Okamoto T, Takenoyama M, Takada K, Katsura M, Suzuki Y, Fujishita T, Kitahara H, Shimamatsu S, Kohno M, Tagawa T, Okano S, Taguchi K, Ichinose Y, Maehara Y. Clinical significance of detecting somatic gene mutations in surgically resected adenosquamous cell carcinoma of the lung in Japanese patients. Ann Surg Oncol 2015; 22: 2593-8.

[33] Watanabe Y, Shiraishi K, Takahashi F, Yoshida A, Suzuki K, Asamura H, Takeuchi M, Furuta K, Tsuta K. Biomarker expression and druggable gene alterations for development of an appropriate therapeutic protocol for pulmonary adenosquamous carcinoma. Histopathology 2015; 66: 939-48.