Insights into the roles of hnRNP A2/B1 and AXL in non-small cell lung cancer

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Received August 13, 2014; Accepted June 2, 2015

DOI: 10.3892/ol.2015.3457

Abstract. Lung cancer has long been one of the most serious types of malignant tumor, and is associated with high incidence and mortality rates. Despite advancements in the comprehensive treatment of the disease, particularly with targeted therapeutic agents, there has been little improvement in the 5-year survival rates of patients. One of the leading causes of mortality in lung cancer is the lack of effective early diagnostic criteria. On this basis, the present study aimed to identify an index with potential in the early diagnosis and prognosis of lung cancer. The current study determined the expression of heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 and AXL proteins in non-small cell lung cancer (NSCLC) tumor samples, and performed prognostic analysis of the collected clinical data to identify any association. In addition, RNA interference was performed to silence the expression of hnRNP A2/B1, allowing evaluation of its molecular and cellular functions, and determination of the mechanism of hnRNP A2/B1 in NSCLC by means of AXL mediation. It was identified that the positive expression rate of hnRNP A2/B1 and AXL proteins were significantly higher in NSCLC compared with paracancerous lung tissues ($P<0.05$). Furthermore, the expression of hnRNP A2/B1 protein was correlated with the expression AXL. Thus, the expression of hnRNP A2/B1 and AXL protein are factors affecting prognosis in patients with NSCLC. Of these, hnRNP A2/B1 appears to be an independent risk factor.

Introduction

Lung cancer has become one of the most serious malignant types of tumor, and is associated with high incidence and mortality rates worldwide. According to a report by the American Cancer Society in 2013, the occurrence of lung cancer constitutes 14% of the total incidence of malignant tumors (1,2). Furthermore, lung cancer is the second most common malignancy globally after prostatic and breast cancer, and accounts for 28 and 16% of mortalities caused by malignancies in men and women, respectively. Thus, lung cancer is the most lethal human malignancy (3). In fact, lung cancer has even higher incidence and mortality rates in China, with ~85% of cases of lung cancer diagnosed as non-small cell lung cancer (NSCLC) (2). This indicates the importance of fundamental investigations into clinical treatment guidance for patients with NSCLC.

Recently, significant advancements have been made in the comprehensive treatment of lung cancer, particularly with targeted agents. Despite this, there has been little improvement in the 5-year survival rates of patients with lung cancer, with the rate remaining as low as 15%. This is true even for those treated patients with surgery, constituting ≤40% of patients (3). One of the leading factors associated with this poor survival is the lack of effective early diagnostic criteria. In fact, the majority of patients are already in the aggressive stage when diagnosed with lung cancer (4). Another important factor is the lack of efficient treatment strategies for severe cases with marked resistance to multiple agents. On this basis, there is an urgent requirement to identify effective diagnostic criteria for the early diagnosis of lung cancer, as well as indexes for its prognosis, that together may contribute to improvements in the total survival rates.

The heterogeneous nuclear ribonucleoprotein (hnRNP) family are important in the process of mRNA transcription, shearing and splicing (5). The superfamily is composed of >20 members, the majority of which bind to the splicing sequences located within introns and exons to perform regulatory roles (6). Among them, the principle members are hnRNP A1, A2, A3 and B1. hnRNP A2 and B1 constitute the core, and are isomers of the same protein derived from the hnRNP A2/B1 gene. Thus, there are similarities in the sequence and function of these two proteins (7-9). Numerous studies have identified abnormally high expression of hnRNP A2/B1 in a variety of tumor types (10-14). According to previous reports, it is well-established that hnRNP A2/B1 serves as an early diagnostic indicator of

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Key words: heterogeneous nuclear ribonucleoprotein A2/B1, AXL receptor tyrosine kinase, non-small cell lung cancer

lung cancer (15-17). However, it remains unclear whether it is effective as a prognostic criterion.

AXL belongs to the receptor tyrosine kinase (RTK) family of proteins (18). AXL, together with two other RTKs (TYRO3 and MER), constitutes the TAM tyrosine receptor sub-family. The three proteins share similar structures and functions (19-21). Structurally, TAM proteins are characterized by two immunoglobulin-like extra-cellular domains and two repeating fibronectin type III cytoplasmic kinase domains (19). They also share the same ligand, Gas6. Previous studies indicated that the activation of TAM, as well as the downstream signal transduction cascades, are key in various cellular functions and behaviors, including cell survival, proliferation, migration and adherence (18). High expression of AXL was also identified in a variety of tumors, and AXL appears to be important in the invasion and metastasis of lung cancer (22). More notably, He *et al* (23) performed RNA interference (RNAi) to reduce the expression of hnRNP A2/B1 in Colo16 cancer cells. Using high-throughput gene chip screening, marked differences were observed in the expression of 123 downstream target genes, including AXL, indicating a potential interaction between hnRNP A2/B1 and AXL.

The present study aimed to determine the expression of hnRNP A2/B1 and AXL in NSCLC and paracancerous lung control samples, as well as perform a prognostic analysis of the collected clinical data to explore any potential association. In addition, RNAi was performed to silence the expression of hnRNP A2/B1, on which basis molecular and cellular functions of hnRNP A2/B1 were evaluated to determine its mechanism in NSCLC, possibly by means of AXL mediation. Written informed consent was obtained from all patients.

Materials and methods

Tissue microarray. The tissue microarray was purchased from the National Biological Chip Center (Shanghai, China) and constructed using 150 resected NSCLC and paracancerous lung tissues samples (two sets of chips with serial sections). Matched cancerous and paracancerous samples were collected from the First Hospital of China Medical University (Shenyang, China) between 2004 and 2007 during surgery for pulmonary lobectomy or total pneumonectomy. Following the exclusion of cases with incomplete data, such as gender, age, tumor size, histological type, TNM American Joint Committee on Cancer classification (24), degree of differentiation, lymph node metastasis and prognostic data, 134 cases were available for prognostic analysis. The most recent follow-up time was July 2012. The study was approved by the ethics committee of the First Affiliated Hospital of China Medical University.

Reagents. Mouse monoclonal anti-human hnRNP A2/B1 (cat no. ab6102) and rabbit polyclonal anti-human AXL (cat no. ab72069) antibodies were purchased from Abcam (Cambridge, UK), and monoclonal mouse anti-human β-actin (cat no. SC-47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Additionally, the UltraSensitive™ SP IHC and MaxVision™ DAB kits were purchased from Maxim Biotech, Inc. (Rockville, MD, USA).

Immunohistochemical streptavidin-peroxidase analysis. The tissue chips were dewaxed with dimethylbenzene two times (15 min each), then washed twice with 100% ethanol (5 min each), once with 95% ethanol (2 min each), once with 85% ethanol (2 min each), once with 75% ethanol (2 min each) and three times with distilled water (3 min each). Endogenous peroxidase was blocked with liquid-A of the UltraSensitive SP IHC kit for 30 min and then washed three times with phosphate-buffered saline (PBS; 3 min each). Antigen retrieval was performed at a high pressure using citric acid for 3 min, cooled to room temperature and washed three times with PBS (3 min each). The non-immune serum (liquid-B, UltraSensitive SP IHC kit) was added prior incubating the chips at 37˚C for 30 min to block non-specific antigens. Excess serum was discarded, primary hnRNP A2/B1 (1:500 dilution) and AXL (1:100 dilution) antibodies were added, and the tissue chips were stored at 4˚C overnight. Secondary antibody (solution-C, UltraSensitive SP IHC kit) was added and incubated at 37˚C for 30 min. After washing three times with PBS (3 min each), the coloring conditions were observed under microscopy by adding solution-D of the UltraSensitive SP IHC kit and the DAB reagents. The reaction was terminated and stained with hematoxylin for 3 min, differentiated with 1% hydrochloric acid ethanol and rinsed in water for 10 min. Dehydration with ethanol was performed in gradients prior to clearing in xylene and mounting with neutral gum.

Classification. The results of the immunohistochemical analysis were classified according to the following criterion: The proportion of positive cells (<30%, 1 point; 30-60%, 2 point; >60%, 3 points) and the color of staining (colorless or light yellow, 1 point; yellow, 2 points; brown, 3 points). The final scores were obtained by multiplying the two integrates and were used to determine the following classifications: 1-2, negative expression; and 3-9, positive expression. All scoring was performed by two independent pathologists and the mean of the scores was used as the final result.

Statistical analysis. Comparisons were performed using t-tests, and correlation analysis between the expression of hnRNP A2/B1 and AXL in the lung tissue samples was conducted using the Pearson's correlation method. The clinical data was analyzed by performing a χ^2-test or Fisher's exact probability. Survival curves were constructed according to the Kaplan-Meier method and analyzed using log-rank tests. Furthermore, univariate and multivariate Cox regression analysis was used to identify independent prognostic factors. SPSS software (version 18.0; IBM SPSS, Armonk, NY, USA) was used to perform all statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein expression levels of hnRNP A2/B1 in NSCLC and paracancerous tissue samples. In the present study, a total of 134 NSCLC and paracancerous tissue chip samples were collected. Immunohistochemical staining was used to identify that hnRNP A2/B1 protein is located in the nucleus and cytoplasm of invasive adenocarcinoma (Fig. 1A) and highly differentiated squamous cell carcinoma (Fig. 1B). High

Table I. Expression of hnRNP A2/B1 in NSCLC and paracancerous lung tissues.

Lung tissue	Total cases, n	hnRNP A2/B1 expression, n		Positive rate, %	P-value
		Negative	Positive		
NSCLC	134	47	87	67.9	0.000[a]
Paracancerous	134	121	13	9.7	

[a]P<0.05. hnRNP, heterogeneous nuclear ribonucleoprotein; NSCLC, non-small cell lung cancer.

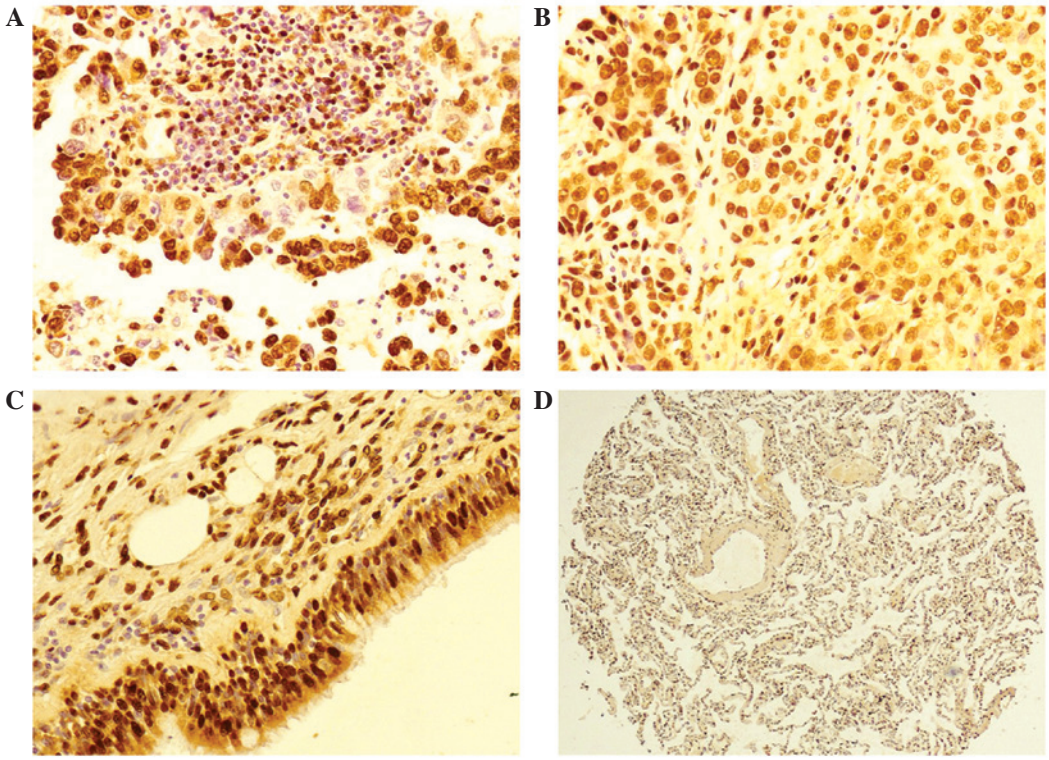

Figure 1. Expression of heterogeneous nuclear ribonucleoprotein A2/B1 in the nucleus and cytoplasm of (A) invasive adenocarcinoma (B) highly differentiated squamous cell carcinoma, (C) hyperplastic bronchial epithelia (magnification, x200) and (D) paracancerous lung tissue samples (magnification, x50; hematoxylin stain).

hnRNP A2/B1 expression was observed in hyperplastic bronchial epithelia (Fig. 1C), however, little expression of hnRNP A2/B1 was observed in the paracancerous lung tissues (Fig. 1D). According to statistical analysis, there was a significantly greater rate of positive hnRNP A2/B1 expression in NSCLC compared with paracancerous lung tissues (P<0.001; Table I).

Association between hnRNP A2/B1 expression and clinicopathological factors. According to statistical analysis of the clinical data, it was identified that the expression of hnRNP A2/B1 was significantly correlated with the differentiation of lesions in 134 cases of NSCLC. The lower the degree of differentiation, the greater the positive expression of hnRNP A2/B1 (P=0.001). Furthermore, according to the TNM classification system, there was a significant increase in positive hnRNP A2/B1 expression in phases III/IV compared with phases I/II (P=0.007). However, hnRNP A2/B1 expression was not significantly associated with gender, age, histological type, lymph node metastasis or tumor size (Table II).

Association between hnRNP A2/B1 expression and survival. To analyze survival, Kaplan-Meier plots were constructed for hnRNP A2/B1-positive and -negative patients (Fig. 2A). The median survival time was 54.7±3.8 months for hnRNP A2/B1-positive patients and 82.4±3.5 months for hnRNP A2/B1-negative patients. There was a statistically significant difference between survival in these two groups (log-rank test, P<0.001). Compared with other clinical factors, such as gender, histological type and degree of differentiation, positive expression of hnRNP A2/B1 was identified as an independent risk factor that may influence the prognosis of patients with NSCLC (P=0.001; Table III), according to multivariate Cox regression analysis.

Protein expression levels of AXL in NSCLC and paracancerous tissue samples. Immunohistochemical analysis of 134 NSCLC

Table II. Association between hnRNP A2/B1 expression and clinicopathological factors in patients with NSCLC.

Clinicopathological factor	Cases, n (n=134)	hnRNP A2/B1 expression -	hnRNP A2/B1 expression +	Positive rate, %	P-value
Gender					
Male	99	35	64	64.6	1.000
Female	35	12	23	65.7	
Age, years					
≤60	58	19	39	67.2	0.716
>60	76	28	48	63.2	
Histological type					
Adenocarcinoma	67	22	45	67.2	0.718
Squamous cell carcinoma	67	25	42	62.7	
Degree of differentiation					
High	23	13	10	43.5	0.001[a]
Middle	73	29	44	60.3	
Low	38	5	33	86.8	
TNM classification					
I/II	99	41	58	58.6	0.013[a]
III/IV	35	6	29	82.9	
Lymphatic metastasis					
Yes	60	20	40	66.7	0.720
No	74	27	47	63.5	
Tumor size, cm					
≤3	48	21	27	56.3	0.133
>3	86	26	60	69.8	

[a]P<0.05. hnRNP, heterogeneous nuclear ribonucleoprotein; NSCLC, non-small cell lung cancer.

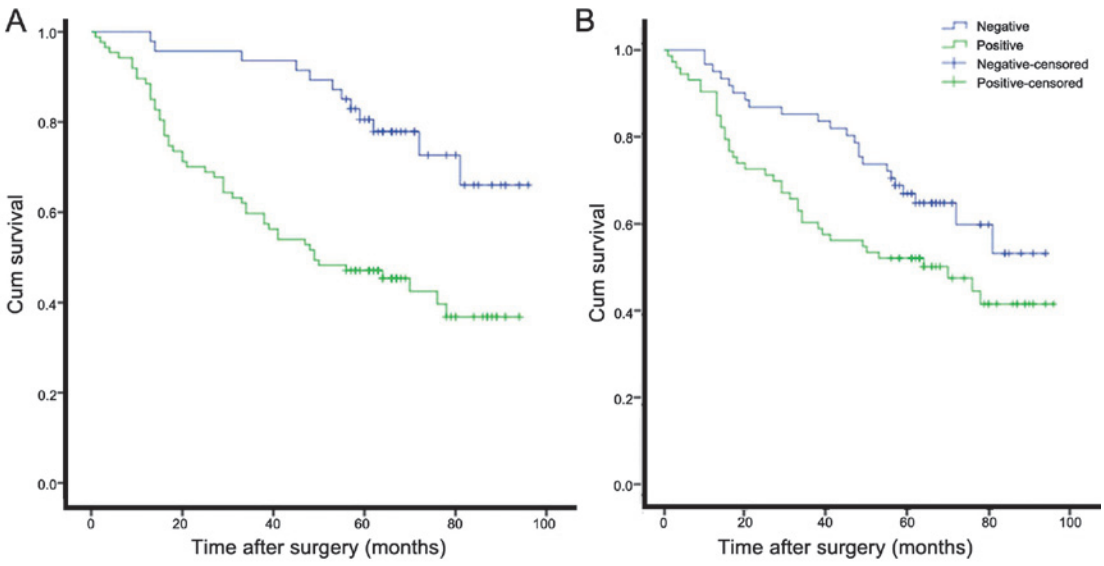

Figure 2. Kaplan-Meier plots for patients with different expression rates of (A) heterogeneous nuclear ribonucleoprotein A2/B1 and (B) AXL. Cum, cumulative.

and paracancerous tissue samples revealed that AXL protein is predominantly located in the cytoplasm (Fig. 3), with little expression in the nucleus. Furthermore, statistical analysis identified a significantly greater rate of positive AXL expression in NSCLC compared with in paracancerous lung tissues (P<0.05; Table IV).

Table III. Univariate and multivariate regression analysis of clinical data and prognosis with Cox model.

	Univariate regression analysis		Multivariate regression analysis	
Index	95% confidence interval	P-value	95% confidence interval	P-value
Gender	0.926 (0.704-1.219)	0.584	-	-
Tumor size	0.756 (0.572-1.000)	0.050[a]	-	-
Histological type	1.083 (0.844-1.390)	0.532	-	-
Degree of differentiation	0.997 (0.687-1.446)	0.986	-	-
TNM classification	1.800 (1.378-2.351)	<0.001[a]	1.558 (1.086-2.237)	0.016[a]
Lymphatic metastasis	2.234 (1.396-3.867)	0.001[a]	1.490 (0.809-2.746)	0.201
Age	1.910 (1.112-3.280)	0.019[a]	2.119 (1.228-3.655)	0.007[a]
hnRNPA2/B expression	0.313 (0.166-0.588)	<0.001[a]	2.928 (1.539-5.573)	0.001[a]

[a]P<0.05. hnRNP, heterogeneous nuclear ribonucleoprotein.

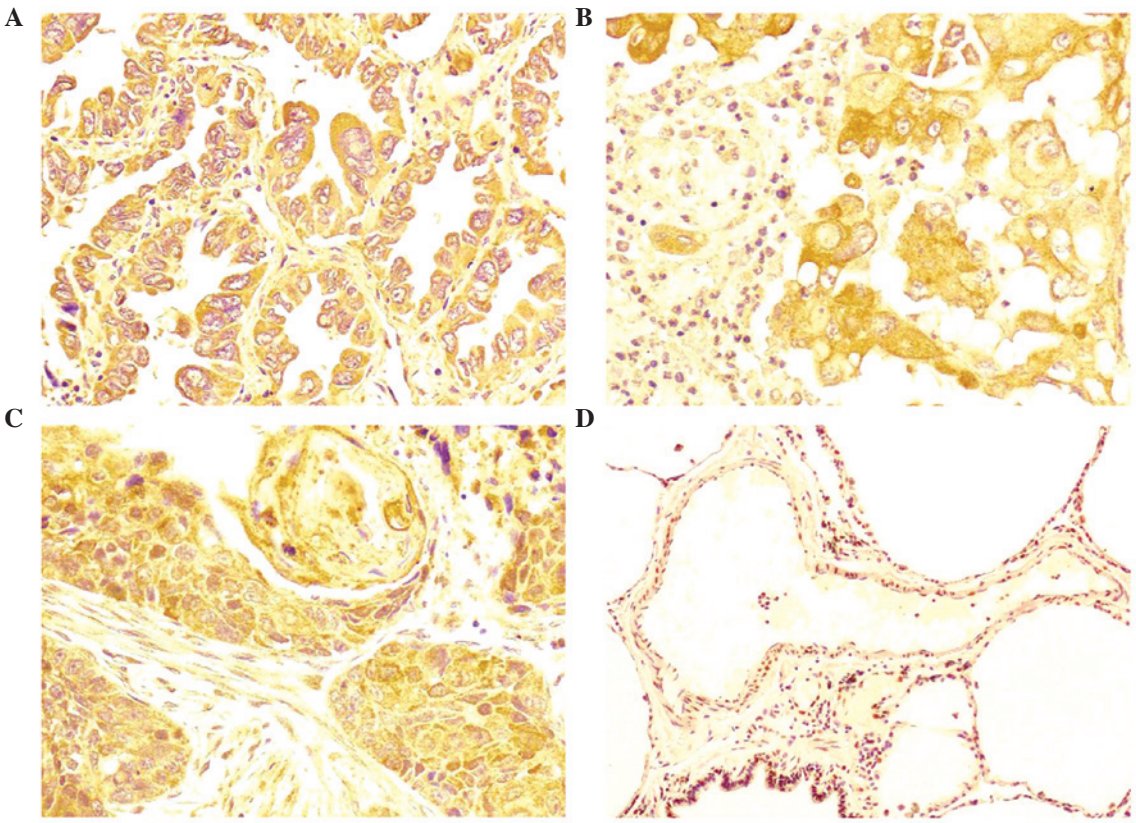

Figure 3. Immnuohistochemical expression of AXL in the nucleus and cytoplasm of (A) highly differentiated adenocarcinoma, (B) low/moderately differentiated adenocarcinoma and (C) highly differentiated squamous cell carcinoma non-small cell lung cancer tissue samples (magnification, x200), and (D) paracancerous lung tissue samples (magnification, x100; hematoxylin stain).

Association between AXL expression and clinicopathological factors. Following statistical analysis of the clinical data, it was identified that the expression of AXL is significantly correlated with the differentiation of lesions in the 134 cases of NSCLC investigated. The lower the degree of differentiation, the greater the positive expression of AXL (P=0.001). According to the TNM classification system, there was a significant increase in the expression of AXL in TNM phases II/III/IV compared with phase I (P=0.005). However, AXL expression was not significantly associated with other clinical factors, such as gender, age, histological type, lymph node metastasis and tumor size (Table V).

Association between AXL expression and survival. To analyze survival, Kaplan-Meier plots were constructed for AXL-positive and -negative patients (Fig. 2B). The median survival time was 58.2±4.3 months for AXL-positive patients and 71.8±3.8 months for AXL-negative patients. There was a statistically significant difference between survival in these two groups (log-rank test; P=0.042). According to univariate

Table IV. Expression of AXL in NSCLC and paracancerous lung tissues.

Lung tissue	Total cases, n	AXL expression, n		Positive rate, %	P-value
		Negative	Positive		
NSCLC	134	61	73	54.5	0.001[a]
Paracancerous	134	95	39	29.1	

[a]P<0.05. NSCLC, non-small cell lung cancer.

Table V. Association between AXL expression and clinicopathological factors in patients with NSCLC.

Clinicopathological factor	Cases, n (n=134)	AXL expression		Positive rate, %	P-value
		-	+		
Gender					
Male	99	42	57	57.6	0.242
Female	35	19	16	45.7	
Age, years					
≤60	58	25	33	56.9	0.727
>60	76	36	40	53.6	
Histological type					
Adenocarcinoma	67	31	36	53.7	1.000
Squamous cell carcinoma	67	30	37	55.2	
Degree of differentiation					
High	23	17	6	26.1	0.001[a]
Middle	73	37	36	49.3	
Low	38	7	31	81.6	
TNM classification					
I	23	17	6	26.1	0.005[a]
II/III/IV	111	44	67	60.4	
Lymphatic metastasis					
Yes	60	23	37	61.7	0.092
No	74	38	36	48.7	
Tumor size, cm					
≤3	48	26	22	45.8	0.167
>3	86	35	51	59.3	

[a]P<0.05. NSCLC, non-small cell lung cancer.

Cox regression analysis, the positive expression of AXL was identified as an independent risk factor that may influence prognosis in NSCLC (P=0.045; Table VI).

Correlation between the expression of hnRNP A2/B1 and AXL. Pearson's correlation of the expression of hnRNP A2/B1 and AXL identified an unexpectedly high correlation, with a coefficient of 0.459 (P<0.001; data not shown). Thus, there was a statistically significant correlation between the expression of hnRNP A2/B1 and AXL in the 134 cases of NSCLC analyzed in the current study. In addition, Fig. 4 indicates the staining patterns of hnRNP A2/B1 and AXL in different cases of NSCLC. It was observed that the expression of hnRNP A2/B1 and AXL correlated well in the same tissue, with correlation increasing as the degree of tissue differentiation reduced.

Discussion

In the present study, the expression of hnRNP A2/B1 was examined in NSCLC and paracancerous tissue samples. The association between hnRNP A2/B1 expression and the prognosis of patients with NSCLC was investigated by analyzing the statistical correlation between hnRNP A2/B1 expression

Table VI. Univariate and multivariate Cox regression analysis of clinical data and prognosis of patients with NSCLC.

	Univariate regression analysis		Multivariate regression analysis	
Index	95% confidence interval	P-value	95% confidence interval	P-value
Gender	0.926 (0.704-1.219)	0.584	-	-
Tumor size	0.756 (0.572-1.000)	0.050[a]	-	-
Histological type	1.083 (0.844-1.390)	0.532	-	-
Degree of differentiation	0.997 (0.687-1.446)	0.986	-	-
TNM classification	1.800 (1.378-2.351)	<0.001[a]	1.733 (1.219-2.464)	0.002[a]
Lymphatic metastasis	2.234 (1.396-3.867)	0.001[a]	1.291 (0.707-2.357)	0.405
Age	1.910 (1.112-3.280)	0.019[a]	2.025 (1.173-3.497)	0.011[a]
AXL expression	1.696 (1.012-2.841)	0.045[a]	1.406 (0.830-2.383)	0.206

[a]P<0.05. NSCLC, non-small cell lung cancer.

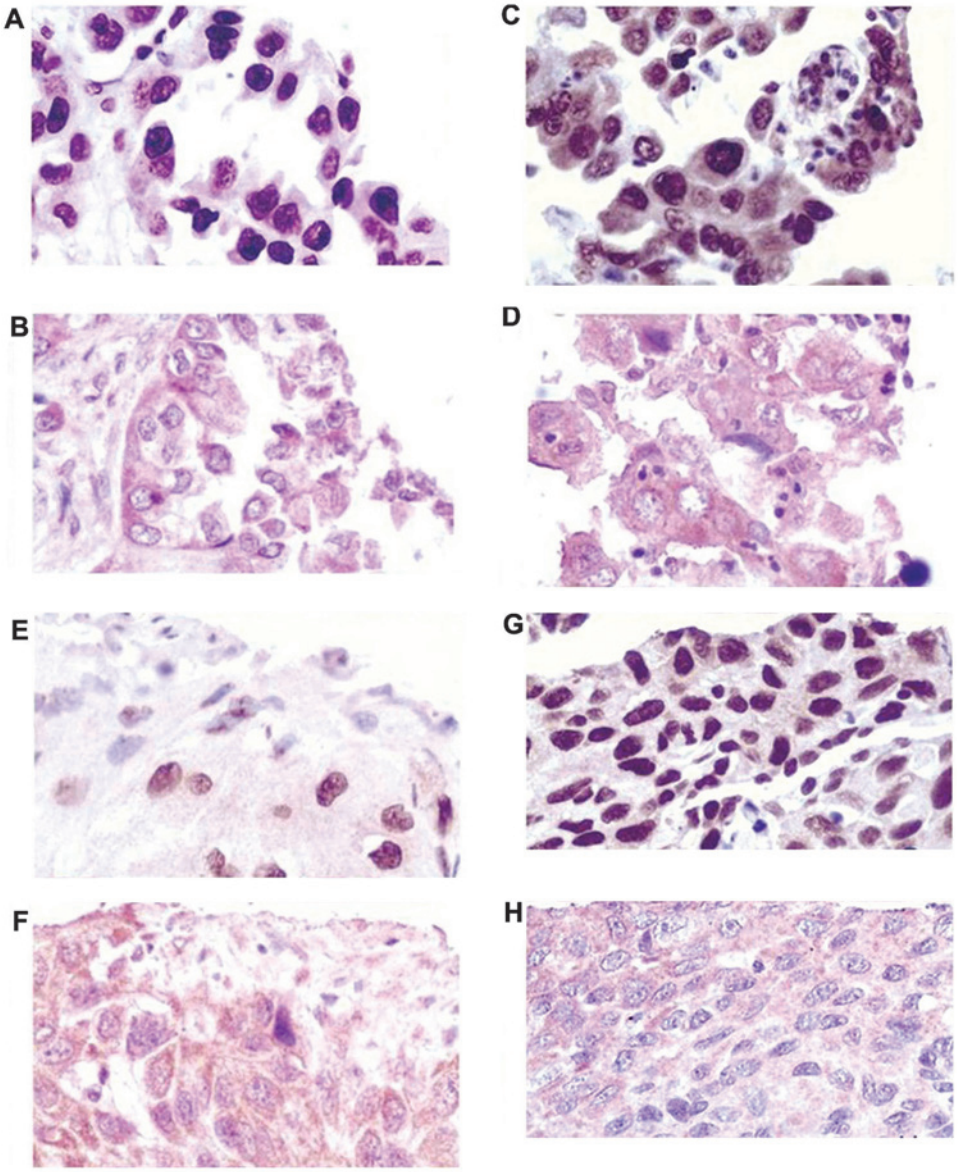

Figure 4. Expression of heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 and AXL in samples of highly and poorly differentiated (A-D) adenocarcinoma and (E-H) squamous cell carcinoma. Staining of hnRNP A2/B1 in (A) highly and (C) poorly differentiated adenocarcinoma; AXL in (B) highly and (D) poorly differentiated adenocarcinoma; hnRNP A2/B1 in (E) highly and (G) poorly differentiated squamous cell carcinoma; and AXL in (F) highly and (H) poorly differentiated squamous cell carcinoma samples (magnification, x400; hematoxylin stain).

and survival, as well as numerous clinicopathological factors.

The present study identified that the expression of hnRNP A2/B1 was predominantly located in the nucleus of cells, followed by the cytoplasm. This indicates the potential transfer of hnRNP A2/B1 from the cytoplasm to the nucleus, which was previously associated with tumor progression (25). The results of tissue microarray assays demonstrated that the rate of positive hnRNP A2/B1 expression was significantly higher in lung tumor than in paracancerous lung tissues. Furthermore, analysis of clinical factors revealed that positive expression of hnRNP A2/B1 was significantly higher in TNM stages III-IV compared with stages I-II (P=0.013). In other words, as NSCLC progresses, hnRNP A2/B1 expression increases. It was also identified that the expression of hnRNP A2/B1 is highly associated with the degree of tumor differentiation. Thus, the lower differentiation, the higher level of positive hnRNP A2/B1 expression. This indicates that the expression of hnRNP A2/B1 increases as the NSCLC becomes more severe.

Based on the aforementioned data, it is proposed that hnRNP A2/B1 may serve as a prognostic factor in NSCLC. This proposal is supported by the results of the current survival analysis. In addition to the log-rank test, Kaplan-Meier survival curves clarified a negative correlation between positive expression of hnRNP A2/B1 and survival rates of patients with NSCLC (P=0.000). Thus, high positive expression of hnRNP A2/B1 indicates an increased risk of patients with NSCLC exhibiting a poor prognosis. By using the multivariate Cox regression model to analyze the data, it was demonstrated that positive expression of hnRNP A2/B1 may be an independent risk factor for the prognosis of NSCLC (P=0.001).

He *et al* (23) previously reported that differences in the expression of 123 downstream target genes in Colo16 squamous carcinoma cells following RNAi silencing of hnRNP A2/B1 expression by means of high-throughput gene chip screening. Among them, AXL expression was affected, indicating a potential interaction between hnRNP A2/B1 and AXL. Similar to the results reported by Linger *et al* (26), the current study revealed that positive expression of AXL was significantly higher in lung tumors than in paracancerous lung tissues. Additional investigation of clinical data indicated that AXL is highly associated with the prognosis of lung cancer, using univariate (but not multivariate) Cox regression models. Abnormally high positive expression of AXL may be involved in the processes of apoptosis and tumor invasion (27), and, thus, associated with the degree of tumor differentiation and clinical TNM stage classification. A pair-wise t-test of protein expression demonstrated a significant correlation between hnRNP A2/B1 and AXL expression in the same tissue microarray (P<0.05).

In conclusion, the present study identified a significant increase in the protein expression of hnRNP A2/B1 and AXL in NSCLC compared with paracancerous lung tissue samples. Thus, hnRNP A2/B1 and AXL constitute independent risk factors for the prognosis of patients with NSCLC. In addition, the results of immunohistochemical analyses indicated a correlation between the expressions of hnRNP A2/B1 and AXL. Therefore, the present study provides the basis for the potential use of hnRNP A2/B1, as well as AXL, as clinicial prognostic criterion in NSCLC.

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