Journal of Thoracic Disease

--Manuscript Summary--

Manuscript ID	JTD-15-444
Title	The BCL11A-XL expression predicts relapse in early stage squamous cell carcinoma and large cell carcinoma
Running Head	BCL11A-XL protein predicts a better outcomes in SCC and LCC
Keywords	non-small cell lung cancer,prognostic,BCL11A-XL
Abstract	Background: The B cell leukemia 11A gene (BCL11A) was identified as a proto-oncogene in hematopoietic cell malignancies and breast cancer. Alternative RNA splicing generates three main transcripts designated as eXtra-Long (XL; 5.9 kb/125 kD), Long (L; 3.8 kb/100 kD) and Short (S, 2.4 kb/35 kD). The BCL11A-XL isoform is not only the largest and the most abundant transcript but also functions as a proto-oncogene of B cell malignancies. Our previous study demonstrated BCL11A was highly expressed in non-small cell lung cancer and was a predict factor of survival and relapse. In the paper we further evaluated the clinical significance of BCL11A isoforms in NSCLC.Materials and methods: The BCL11A isoforms were detected with immunohistochemistry method (IHC) in non-small cell lung cancer with in a cohort (n = 40) of BCL11A overexpression NSCLC patients. Relationship between BCL11A isoforms and the clinicopathological parameters were analyzed.Results: All 40 cases were BCL11A overexpression including 27 squamous cell carcinomas, 8 large cell carcinomas and 5 adenocarcinomas. Compare to the BCL11A-L and S isoforms, the BCL11A-XL isoform was specifically expressed in SCC and LCC (p=0.006). There were 19 (19/40, 47.5%) cases positive for BCL11A-XL expression, SCC accounted for 63.2% (12/19) and LCC accounted for 36.8% (7/19). The survival analysis indicated that BCL11A-XL expression was an independent prognostic factor for DFS (hazards ratio [HR] 0.246; 95% confidence interval [CI] 0.065â€“0.939, p= 0. 040) but not for OS in patients with SCC and LCC.Conclusions Our results demonstrated that the BCL11A-XL isoform might be a potential prognostic biomarker of SCC and LCC.
Section Title	Original Articles

Title: The BCL11A-XL expression predicts relapse in early stage squamous cell carcinoma and large cell carcinoma

Author names:

Na Zhang; Ben-Yuan Jiang; Xu-Chao Zhang; Zhi Xie; Jian Su; Qi Zhang; Jie-Fei Han; Hai-Yan Tu; Yi-Long Wu.

Author Affiliations:

Southern Medical University, Guangzhou, China (Na Zhang); Guangdong Lung Cancer Institute, Guangdong General Hospital & Guangdong Academy of Medical Sciences, Guangzhou, Guangdong, China (Na Zhang, Ben-Yuan Jiang, Xu-Chao Zhang, Zhi Xie, Jian Su, Hai-Yan Tu, Qi Zhang, Jie-Fei Han, Yi-Long Wu)

Corresponding Author: Yi-Long Wu, MD, FACS

Postal address: Guangdong Lung Cancer Institute, Guangdong General Hospital, 106 Zhongshan 2nd Road, Guangzhou 510080, China

Tel: +8620, 83877855

Fax：+8620,83827712

Email: syylwu@live.cn

Abstract

The B cell leukemia 11A gene (BCL11A) was identified as a proto-oncogene in hematopoietic cell malignancies and breast cancer. Alternative RNA splicing generates three main transcripts designated as eXtra-Long (XL; 5.9 kb/125 kD), Long (L; 3.8 kb/100 kD) and Short (S, 2.4 kb/35 kD). The BCL11A-XL isoform is not only the largest and the most abundant transcript but also functions as a proto-oncogene of B cell malignancies. Our previous study demonstrated BCL11A was highly expressed in non-small cell lung cancer and was a predict factor of survival and relapse. In the paper we further evaluated the clinical significance of BCL11A isoforms in NSCLC. This study detected the BCL11A isoforms with immunohistochemistry method (IHC) in non-small cell lung cancer with in a cohort (n = 40) of BCL11A overexpression NSCLC patients. Relationship between BCL11A isoforms and the clinicopathological parameters were analyzed. All 40 cases were BCL11A overexpression including 27 squamous cell carcinomas, 8 large cell carcinomas and 5 adenocarcinomas. Compare to the BCL11A-L and S isoforms, the BCL11A-XL isoform was specifically expressed in SCC and LCC (p=0.006). There were 19 (19/40, 47.5%) cases positive for BCL11A-XL expression, SCC accounted for 63.2% (12/19) and LCC accounted for 36.8% (7/19). The survival analysis indicated that BCL11A-XL expression was an independent prognostic factor for DFS (hazards ratio [HR] 0.246; 95% confidence interval [CI] 0.065–0.939, p= 0. 040) but not for OS in patients with SCC and LCC. Our results demonstrated that the BCL11A-XL isoform might be a potential prognostic biomarker of SCC and LCC.

Keywords: BCL11A-XL, Non-small cell lung cancer, Prognostic

Introduction

BCL11A (B-cell lymphoma/leukemia) also called CTIP1(chicken ovalbumin upstream

promoter transcription factor interacting protein 1), homologous to the mouse Evi9 (Ecotropic viral integration site 9) gene，locates in the human 2p16. *Evi9* was first reported in murine BXH2 leukemia and encodes three isoforms (Evi9a, Evi9b and Evi9c) of a novel zinc finger protein in 2000 by Takuro Nakamura et al. Evi9a (773 amino acids) contains two C2H2-type zinc finger motifs, a proline-rich region, and an acidic domain; Evi9b (486 aa) lacks the first zinc finger motif and part of the proline-rich region; Evi9c (239 aa) lacks all but the first zinc finger motif. The function study showed that Evi9a and Evi9c had the transforming activity for NIH 3T3 cells, but not Evi9b. The highly expression of Evi9 and the isoforms transforming potential in myeloid leukemogenesis suggest that Evi9 might act as a dominant oncogene. After then, the human EVI9 was detected at high levels in brain, spleen, and testis and located at the p13 region on chromosome 2. Also, the function study showed that EVI9 regulated the cellular differentiation in leukemogenesis. Some cases of human lymphoblastic leukemia related to chromosomal translocation t(2;14)(p13;q32) and the comparative genomic hybridization analysis approved that 2p13 abnormalities associated with human malignant lymphoma. According to these findings, the BCL11A gene was considered as a proto-oncogene of malignant hematopoietic diseases also further studies showed that it is essential for pre-B-cell development, lymphocyte maturation, and goblin switching. The BCL11A gene also has three transcripts: BCL11A-XL, BCL11A-L and BCL11A-S. The BCL11A-L and S isoforms show 98.7% identity and 99.2% similarity to the mouse Evi9-a and Evi9-c. BCL11A-XL isoform was specifically generated in human and was restricted expression in bone marrow, lymphoid tissue and brain. Also BCL11A-XL expressed in a range of tumor-derived cell lines, such as primary mediastinal B-cell lymphoma (PMBLs), Germinal center B-cell diffuse large B-cell lymphoma (GCB-DLBCLs) and Activated B-cell diffuse large B-cell

lymphoma (ABC-DLBCLs). Function studies showed BCL11A-XL was a DNA-sequence-specific transcriptional repressor that associated with itself and with other BCL11A isoforms, as well as with the BCL6 proto-oncogene. So BCL11A-XL might play an essential role in tumor development.

BCL11A involvement in solid tumors has been rarely reported. Khaled et al reported BCL11A becomes an oncogene of triple-negative breast cancer and its overexpression promotes tumor formation. Our previous results also demonstrated that BCL11A expression levels were specifically upregulated in NSCLC tissues, especially in squamous cell carcinoma and large cell carcinoma. Multivariate analysis showed that BCL11A was an independent prognostic factor for both disease-free survival (DFS) and overall survival (OS). We investigated the isoforms of BCL11A in NSCLC in the process of function study and analysed the relationship between isoforms and clinicopathological parameters.

Materials and methods

2.1 Tissue samples and clinicopathological characteristics

Specimens were selected from BCL11A overexpression cases and obtained informed consent from 40 NSCLC cases (27 squamous cell lung cancer, 8 large cell lung cancer and 5 adenocarcinoma) who underwent potentially curative surgery at Guangdong Lung Cancer Institute between 2003 and 2008. Every sample prepares 7 sections, one section for pathological assessment, the other 3 for negative control. Hemotoxylin and eosin (H&E) staining was performed on sections of each tissue to determine the percentage of tumor cells by two independent pathologists. Only those samples with tumor content≥80% were allowed to enter this study. This study was approved by the Institutional Review Board (IRB) of Guangdong General Hospital. The staging and histological classifications were based on the World Health Organization (WHO) system. A follow-up evaluation was performed according to standard follow-up protocol. The median

follow-up period was 73.9 months (range, 3.27-130.1 months).

2.2 Immunohistochemistry antibodies

The primary mouse monoclonal antibody BCL11A/123 (Active Motif, USA) was raised against a recombinant protein corresponding to amino acids 637-835 of BCL11A-XL isoform protein. The other two primary mouse monoclonal antibodies BCL11A (ab19487 and ab18688; Abcam, USA) were applied against human BCL11A-L and S isoforms. The ab19487 antibody which epitope is in core of amino acids 172-434 can identify the BCL11A-XL and L isoforms. We can use the exclusive method to distinguish the two isoforms. The ab18688 antibody which epitope is in core of amino acids 1-171 can identify all the three isoforms. The previous study showed that the S isoform locates in cytoplasm while the XL and L are both in the nucleus. So in theory we can distinguish the three isoforms from each other. The second antibody was Mouse IgG (GeneTex, USA) labeled by enzyme horseradish peroxidase.

2.3 Immunohistochemistry on tissue samples

Immunohistochemical staining process was performed according to the protocol provided by DAKO (DakoCytomation, Glostrup, Denmark). Primary antibodies were applied to the sections at a dilution of 1:100 at 4℃ temperature overnight. Those in the control group underwent the same way of add PBS. The sections were counterstained with Harris's hematoxylin. Each tumor was assigned a score according to the intensity of the nucleic or cytoplasmic staining (0 = no staining , 1 = weak staining, 2 = moderate staining, and 3 = strong staining) and the proportion of stained tumor cells (0 = 0%, 1 = 1–10%, 2 = 11–50% , 3 =51–80%, and 4 = 81–100%), as judged by two pathologists, independently. The final immunoreactive score was determined by multiplying the intensity scores by the extent of positivity scores of stained cells, with a minimum score of 0 and a maximum score of 12. Tumors with scores≥2 were classified into the positive BCL11A isoforms

expression group, while the others were classified into the negative group.

2.4 Statistical analysis

All analyses were performed using SPSS 13.0 software. The chi-square test was used to compare qualitative variables, and those with an expected frequency of < 5 were analyzed by Fisher's exact test. A non-parametric test was used to analyze quantitative data. Chi-square tests were used to assess the association of BCL11A-XL level with clinical variables. Survival curves between subgroups divided according to BCL11A-XL expression level were analyzed using the Kaplan-Meier method, and significant differences among subgroups were compared by log-rank test. A multivariate analysis was performed using the stepwise method. Hazards ratios and 95% confidence intervals were calculated using Cox proportional hazards models. P values < 0.05 were considered statistically significant.

Results

3.1 The isoforms of the BCL11A in NSCLC tissues

In our study we found that the BCL11A-XL isoform differentially expressed in squamous cell carcinoma (SCC) and large cell carcinoma (LCC). For the total 40 cases, 19 (47.5%) cases had positive BCL11A-XL expression and 21 (52.5%) cases had negative BCL11A-XL expression. For the 19 BCL11A-XL positive patients, SCC accounts for 63.2% (12/19), LCC accounts for 36.8% (7/19) and no positive case for AC (Table 1). The subcellular location was nucleus, which is accordance with other study results (Figure 1A-C). The BCL11A-L and S isoforms were positive for all 40 cases with no histology difference (data not shown).

3.2 Correlation analysis between the expression of BCL11A-XL and patient clinicopathological characteristics

According to the isoforms result, the BCL11A-XL isoform was histological differentially

expressed. So the correlation analysis was performed to explore whether BCL11A-XL expression was related to clinicopathological variables in patients with NSCLC. The result showed that BCL11A-XL expression had no relationship with gender, smoking status, histology, clinical stage and lymph node status, but it did correlate with histology (p=0.006) and smoking status (p=0.049) (Table 1).

3.3 BCL11A-XL protein expression correlates with disease-free survival (DFS) in early stage patients with NSCLC

For all 40 patients subjected to immunohistochemical staining for BCL11A-XL, 19 (47.5%) had positive BCL11A-XL expression and 21 (52.5%) had negative BCL11A-XL expression. In all patients, the DFS and OS were both no statistically significant difference between BCL11A-XL positive and negative groups (p>0.1) (Figure 2A-B). However, in the subgroup of patients with SCC and LCC, BCL11A-XL expression was predictive of better DFS ($\chi 2$=5.32, P=0.021) (Figure 2C). The median DFS of subgroup patients without BCL11A-XL expression was 20.8 months, but in the BCL11A-XL expression group, only 36.8% of patients relapsed at the endpoint of follow-up. Although no relationship between BCL11A-XL expression and overall survival (OS) was found in SCC and LCC patients, there was a tendency towards decreased survival in patients whose tumors lack of BCL11A-XL (p>0.1) (Figure 2D). The cox regression survival analysis indicated that BCL11A-XL expression was an independent marker of DFS (hazards ratio [HR] 0.246; 95% confidence interval [CI] 0.065–0.939, p= 0. 040) in patients with NSCLC, but not for OS.

Discussion

The current study analysed the isoforms of BCL11A in NSCLC. The result demonstrated that BCL11A-XL protein differentially expressed in SCC and LCC. Statistical analyses demonstrated that

BCL11A-XL expression was strongly associated with histology and smoking status with NSCLC. Moreover, the survival analysis found that patients with BCL11A-XL expression had better DFS outcomes.

The *BCL11A* gene was first detected in B-cell chronic lymphocytic leukemia and considered as a proto-oncogene of malignant hematological diseases. Jiang et al reported for the first time the role of BCL11A overexpression in predicting survival and relapse in non-small cell lung cancer. And this is the first description of BCL11A-XL isoform in lung cancer and its function as a prognostic factor for disease-free survival. Pulford et al detected the BCL11A-XL isoform in both normal and malignant tissues. The result showed BCL11A-XL expression was only observed in CD20-positive B cells and in a variety of B-cell tumors but was undetectable in the myeloma cases. The survival analysis also showed that there was a tendency towards decreased survival in patients whose tumors lacked BCL11A-XL.

Previous studies also showed BCL11A could interact directly with COUP-TF II, *BCL6* and also binds to a GC-rich motif and represses transcription of a downstream reporter gene. We demonstrated that the BCL11A-XL protein was an independent prognostic factor of disease-free survival for SCC and LCC patients. It is also possible that *BCL11A* could interact with the upper proteins and control the regulation of transcriptional networks in cells of NSCLC.

Compare to the previous study, our study demonstrated the similar results. The *BCL11A* gene has three isoforms and all of them associated to malignancies. Compare to the other two isoforms, the BCL11A-XL was differently expressed in NSCLC tissues. It is possible that BCL11A-XL acts as an oncogene in NSCLC. In the future, we might do further researches to explore the BC11A-XL function in non-small cell lung cancer cells.

It is also need to explore the potential mechanisms of abnormal BCL11A activation in

NSCLC. Luo et al. revealed that the BCL11A high expression in Burkitt lymphoma cell line (NAB-2) was associated with Epstein–Barr virus integratation in human genome chromosome 2p13. Many studies also reported that a subset of pulmonary squamous-cell carcinomas and adenocarcinomas show EBV associated, especially lymphoepithelioma-like carcinoma. Our current findings show that BCL11A-XL was aboundant in SCC and LCC, further studies might need to examine whether EBV associate with high expression of BCL11A in lung cancer.

In summary, we report the BCL11A isoforms in NSCLC at the protein level for the first time. Thus, further studies needed to detect the isoforms at the level of RNA and demonstrate whether they could function as oncogenes or tumor suppressor genes in NSCLC. Also we should collect more patients to analyse the relationship between BCL11A-XL and prognosis.

Conclusions

Activation of the BCL11A-XL may be a potential prognostic biomarker of NSCLC, and the BCL11A-XL isoform may play an important role in the tumorigenesis of SCC and LCC.

Acknowledgements

This work was supported by the following funds: Guangdong Provincial Key Laboratory of Lung Cancer Translational Medicine (Grant No. 2012A061400006).

Conflict of interest

The authors declare no conflict of interest.

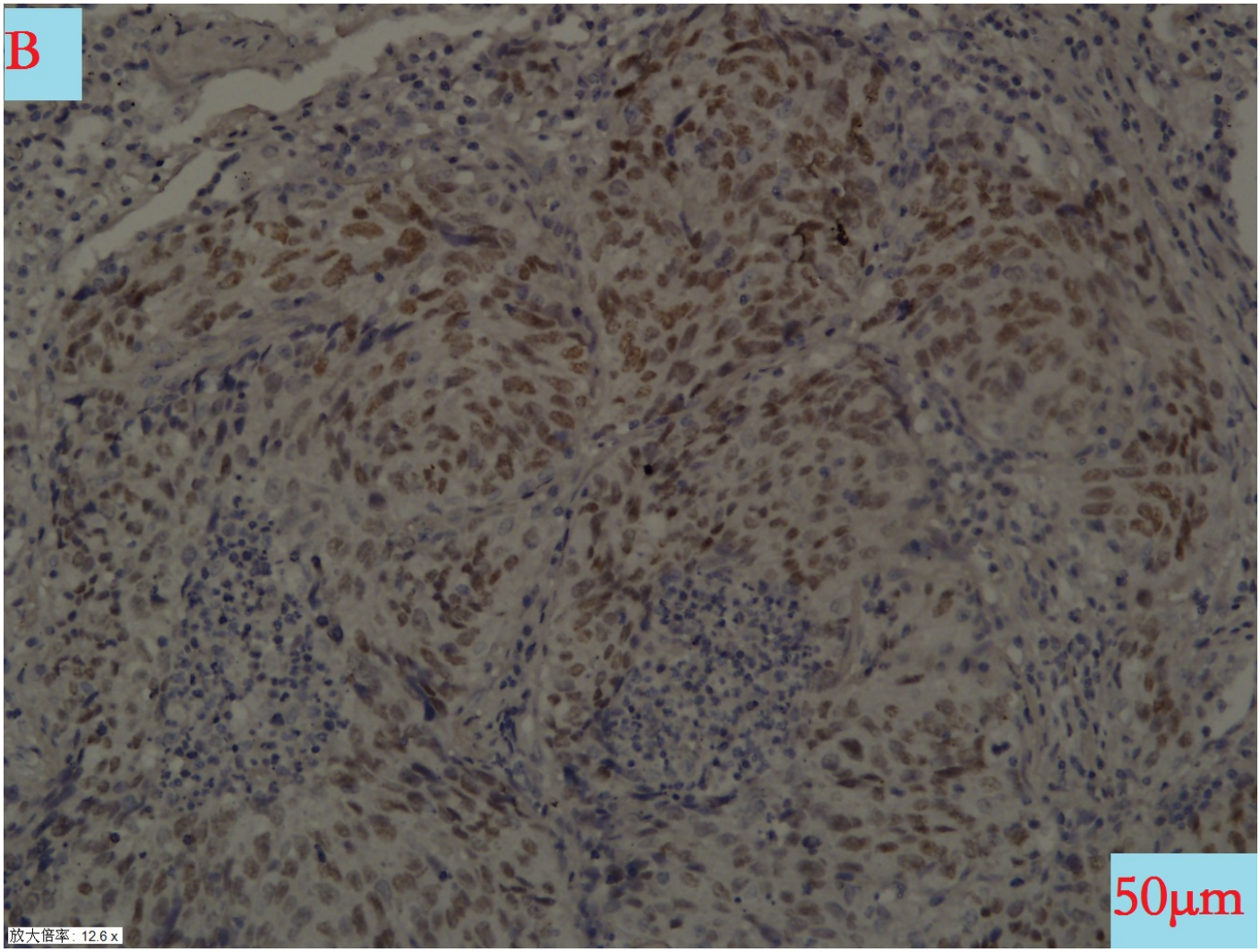
References

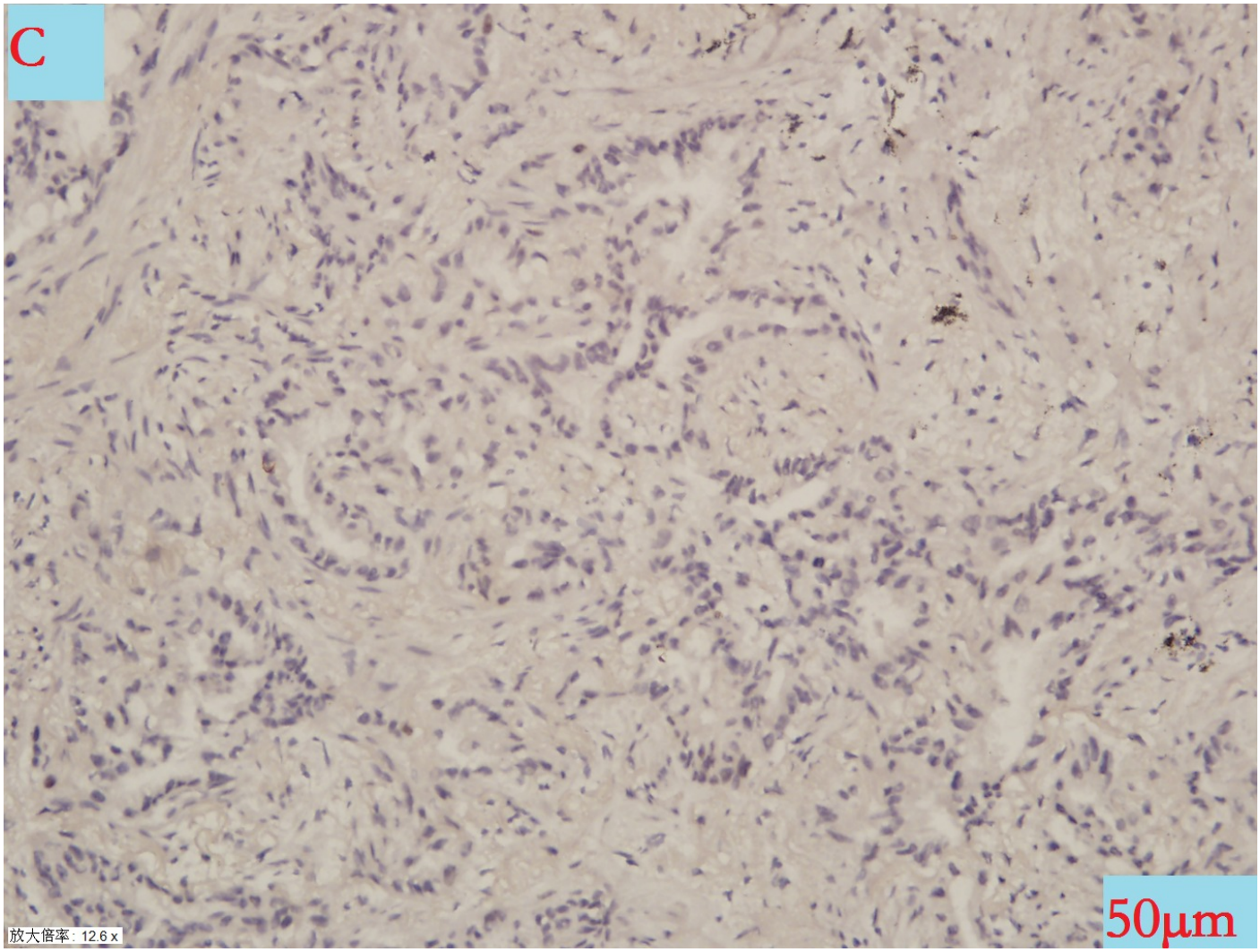
Figure Captions

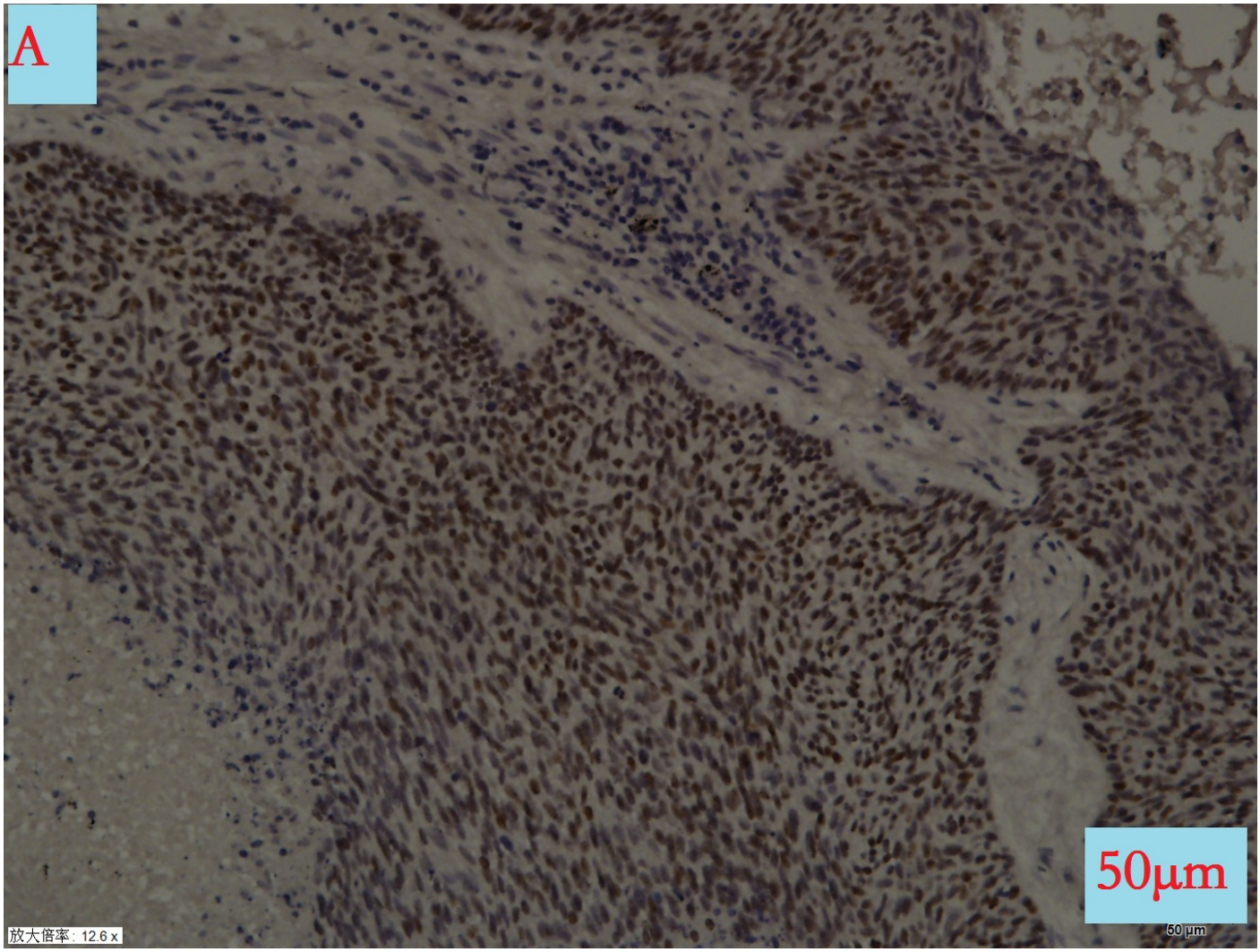
Figure 1 Detection of BCL11A isoforms by immunohistochemical staining in NSCLC cancer. Representative images of (A-B) positive staining of BCL11A-XL isoform in SCC and LCC, and (C) negative staining in AC.

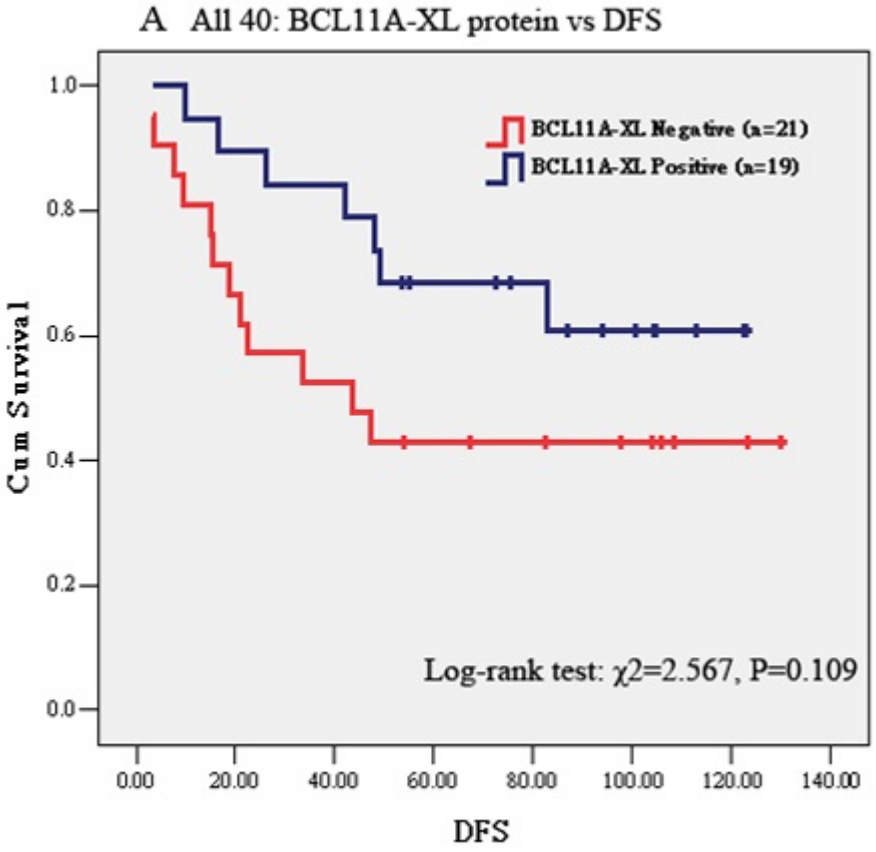
Figure2 Kaplan–Meier curves of NSCLC patients according to BCL11A-XL protein: DFS and OS in all patients (A-B), DFS and OS in subgroup SCC and LCC patients (C-D). P values were calculated by log rank tests.

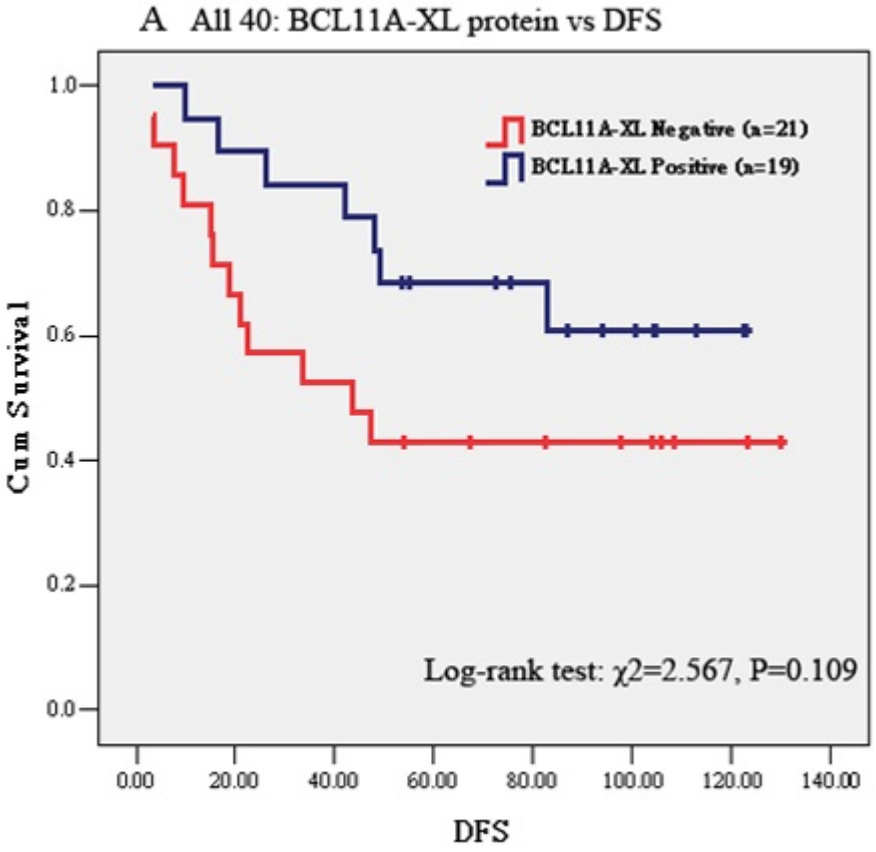
Table 1：Relationships between BCL11A-XL expression and clinicopathological factors.

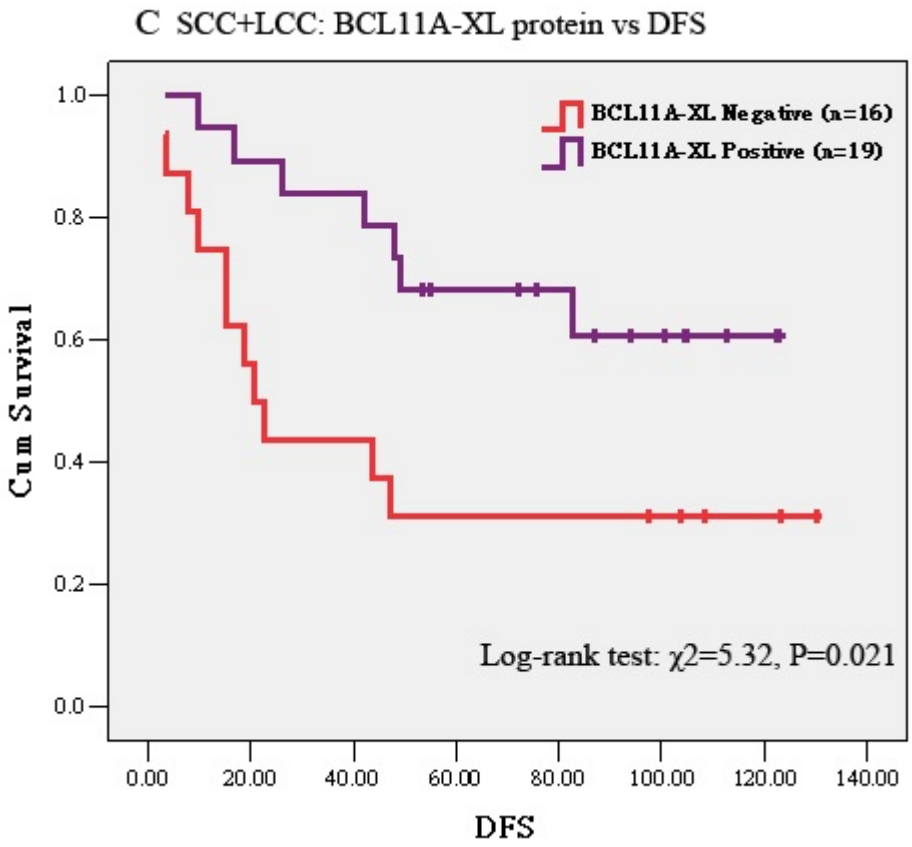

B
50μm
放大倍率: 12.6 x

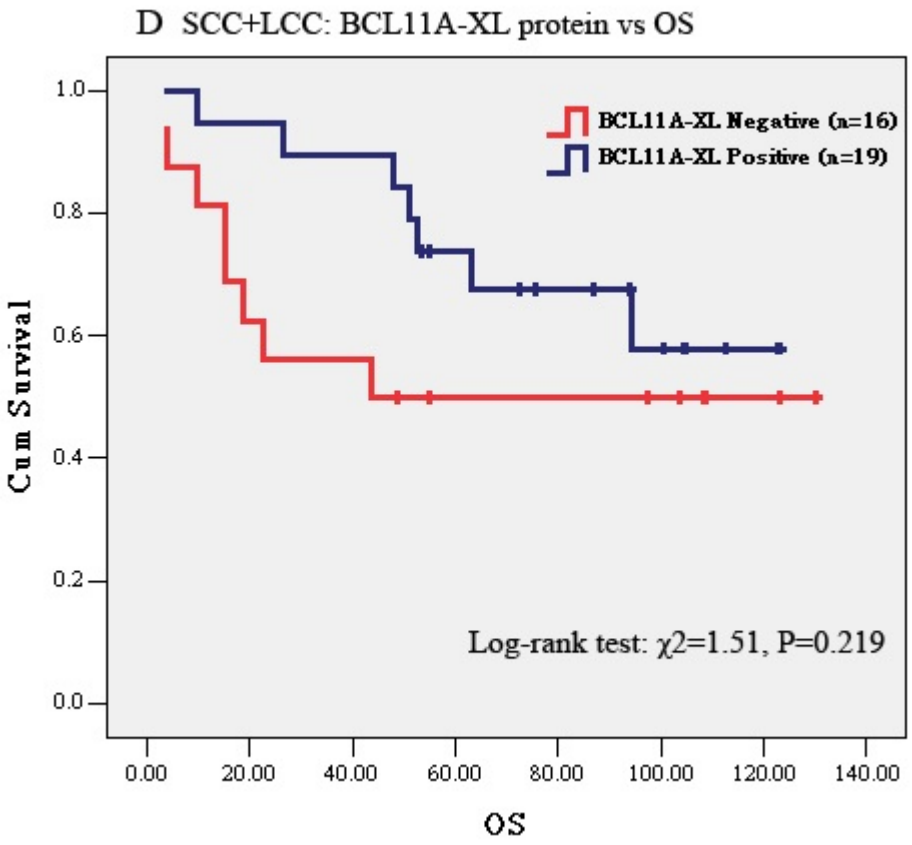

C
放大倍率: 12.6 x
50μm


A
50μm
放大倍率: 12.6 x
50 μm


A All 40: BCL11A-XL protein vs DFS
BCL11A-XL Negative (n=21)
BCL11A-XL Positive (n=19)
Log-rank test: χ2=2.567, P=0.109
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
DFS


A All 40: BCL11A-XL protein vs DFS
BCL11A-XL Negative (n=21)
BCL11A-XL Positive (n=19)
Log-rank test: χ2=2.567, P=0.109
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
DFS


C SCC+LCC: BCL11A-XL protein vs DFS
BCL11A-XL Negative (n=16)
BCL11A-XL Positive (n=19)
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
Log-rank test: χ2=5.32, P=0.021
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
DFS


D SCC+LCC: BCL11A-XL protein vs OS
BCL11A-XL Negative (n=16)
BCL11A-XL Positive (n=19)
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
Log-rank test: χ2=1.51, P=0.219
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
OS

	BCL11A-XL		
Parameters	Positive	Negative	*p* value
Histology type			0.006
SCC	12 (44.4%)	15 (55.6%)	
LCC	7 (87.5%)	1 (22.5%)	
AC	0 (0.0%)	5 (100.0%)	
Smoking status			0.049
NO	8 (72.7%)	3 (14.3%)	
Yes	11 (37.9%)	18 (62.1%)	
Gender			0.085
Male	14 (41.2%)	20 (58.8%)	
Femal	5 (83.3%)	1 (16.7%)	
Stage			0.370
Ⅰ	11 (57.9%)	15 (71.4%)	
Ⅱ-Ⅲ	8 (42.1%)	6 (28.6%)	
Lymph node status			0.583
0	13 (44.8%)	16 (55.2%)	
1-2	6 (54.5 %)	5 (45.4%)	
Age			0.726
<=65	11 (50.0%)	11 (50.0%)	
>65	8 (44.4%)	10 (55.6%)	