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Original Article

Clinicopathological significance and prognostic value of DIXDC1 and β-catenin in NSCLC

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Abstract: Objective: To investigate the clinicopathological and prognostic significance of DIXDC1 and β-catenin in non-small cell lung cancer (NSCLC). Methods: 93 cases of tissue microarray samples for NSCLC were analyzed by immunohistochemistry using DIXDC1 and β-catenin antibodies. Then, correlation of DIXDC1 and β-catenin expression with clinicopathological features and prognosis of NSCLC patients was statistically analyzed. Results: DIXDC1 overexpression was detected in 62.4% (58/93) of NSCLCs, which was significantly correlated with nodal metastasis and pathological stage ($P<0.01$). The aberrant β-catenin expression was detected in 63.4% (59/93) of NSCLCs, there is a positive association between DIXDC1 and β-catenin expression. Furthermore, co-expression of DIXDC1 and β-catenin predicts a poor prognosis and acts as an independent prognostic factor. Conclusion: co-expression of DIXDC1 and β-catenin has an independent predictive value of poor prognosis in NSCLC, DIXDC1 might stabilize β-catenin and promote malignant progression of NSCLC.

Keywords: DIXDC1, β-catenin, NSCLC, prognosis

Introduction

Lung cancer is a serious threat to public health, ranking number 1 in cancer-related deaths throughout the world [1]. Non-small cell lung cancer (NSCLC), mainly including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma, accounts for approximately 85% of lung cancers. Despite progress in the multimodality treatment of lung cancer in recent years, the survival rate of patients with lung cancer remains low, with a 5-year overall survival of merely 15% [2, 3]. Therefore, there is an urgent need for the determination of useful prognostic molecular markers for clinical management of patients with NSCLC.

β-catenin is a key component of the canonical Wnt pathway that plays pivotal roles in embryogenesis, differentiation and tumorigenesis [4]. Wnt signaling is transduced by β-catenin, which is regulated by the adenomatous polyposis coli (APC)/Axin/glycogen synthase kinase (GSK)-3β complex. At the plasma membrane, β-catenin is associated with the cadherin class of cell-adhesion proteins and regulates cell adhesion. In the presence of Wnt signal, β-catenin phosphorylation by GSK-3β is inhibited, resulting in the accumulation of free cytosolic β-catenin. The elevated β-catenin can translocate to the nucleus, where it binds T-cell factor/lymphoid enhancer factor (TCF/Lef) and activates its target gene expression [5, 6].

DIX domain containing 1 (DIXDC1) is a recently identified human homolog of a zebrafish Coiled-coil-DIX1 (Ccd1) gene that acts a positive regulator of the Wnt signaling pathway functioning downstream of Wnt and upstream of Axin [7, 8]. Recent studies have shown that DIXDC1 promotes the proliferation of nerve cells and neuronal differentiation by modulating GSK3-dependent β-catenin phosphorylation [9, 10]. In the field of cancer research, Wang et al. reported that the elevated expression of DIXDC1 promoted the proliferation of colon cancer cells and invasion of gastric cancer cells through classical β-catenin dependent Wnt pathway [11, 12]. Xu et al. reported DIXDC1 increased invasion and migration ability of non-small-cell lung cancer cells via the PI3K/AKT pathway [13]. However, clinicopathological significance and prognostic values of DIXDC1 and β-catenin proteins remain unclear in NSCLC.

Table 1. Relationship between clinicopathologic features and immunohistochemical staining results

		DIXDC1			β-catenin		
Characteristics	n	Negative	Positive	p	Negative	Positive	p
Total	93	35	58		34	59	
Age							
<60 years of age	38	13	25		10	28	
≥60 years of age	55	22	33	0.571	24	31	0.088
Sex							
Male	65	25	40		27	38	
Female	28	10	18	0.802	7	21	0.129
Smoking history							
Yes	49	19	30		19	30	
No	44	16	28	0.811	15	29	0.640
Tumor size							
<5 cm	55	23	32		19	36	
≥5 cm	38	12	26	0.316	15	23	0.628
Histology							
Squamous	43	16	27		18	25	
Adenocarcinoma	50	19	31	0.937	16	34	0.325
Grade							
Well/moderately	77	31	46		27	50	
Poorly differentiated	16	4	12	0.252	7	9	0.512
T status							
T1, T2	66	29	37		24	42	
T3, T4	27	6	21	0.050	10	17	0.951
Nodal invasion							
No	49	29	20		19	30	
Yes	44	6	38	0.000*	15	29	0.640
p Stage							
I, II	59	30	29		21	38	
III, IV	34	5	29	0.001*	13	21	0.799

*represents statistical significance.

In this study, a tissue microarray covered 93 patients was used to evaluate protein expression of DIXDC1 and β-catenin by immunohistochemical staining. Associations between DIXDC1 and β-catenin protein expression as well as with clinicopathological parameters and patients survival were analyzed by statistical methods.

Materials and methods

Case selection and tissue microarray construction

A total of 93 paraffin blocks incorporating tumor samples and corresponding paracancerous tissues samples were available from patients with resectable NSCLC who had undergone surgery. Written informed consent for the tissue specimens was received from all participants, and the study was approved by the local ethical committee. All patients were followed after surgery until 31 July 2015 with detail clinicopathological data including age, gender, smoke history, tumor size, histological classification and grading, lymph node and distal metastasis, pathological stage and overall survival. The survival time ranged from 3 to 102 months (average, 29 months).

Tissue microarrays (TMA) were constructed using diameter of 1.5-mm cores. The representative of tumor tissues and the corresponding tissues adjacent to carcinoma were selected by the pathologists. TMA blocks were constructed using an automated tissue arrayer (Beecher Instruments, Sun Prarie, WI). The array blocks were cut into five-micron sections, and one section was stained with hematoxylin-eosin to verify presence of tumor cells.

Immunohistochemistry and scoring

Immunohistochemical method (Envision™ two-step method) was used to detect the protein expression of DIXDC1 and β-catenin in NSCLC tissue microarray. Tissues sections were deparaffinized with xylene, rehydrated with graded alcohol. Antigen retrieval for DIXDC1 and β-catenin was performed in 0.1 M citrate buffer at pH 6.0 for 10 min followed by blocking endogenous peroxidases and incubation with 5% normal mouse serum for 10 min. Then sec-

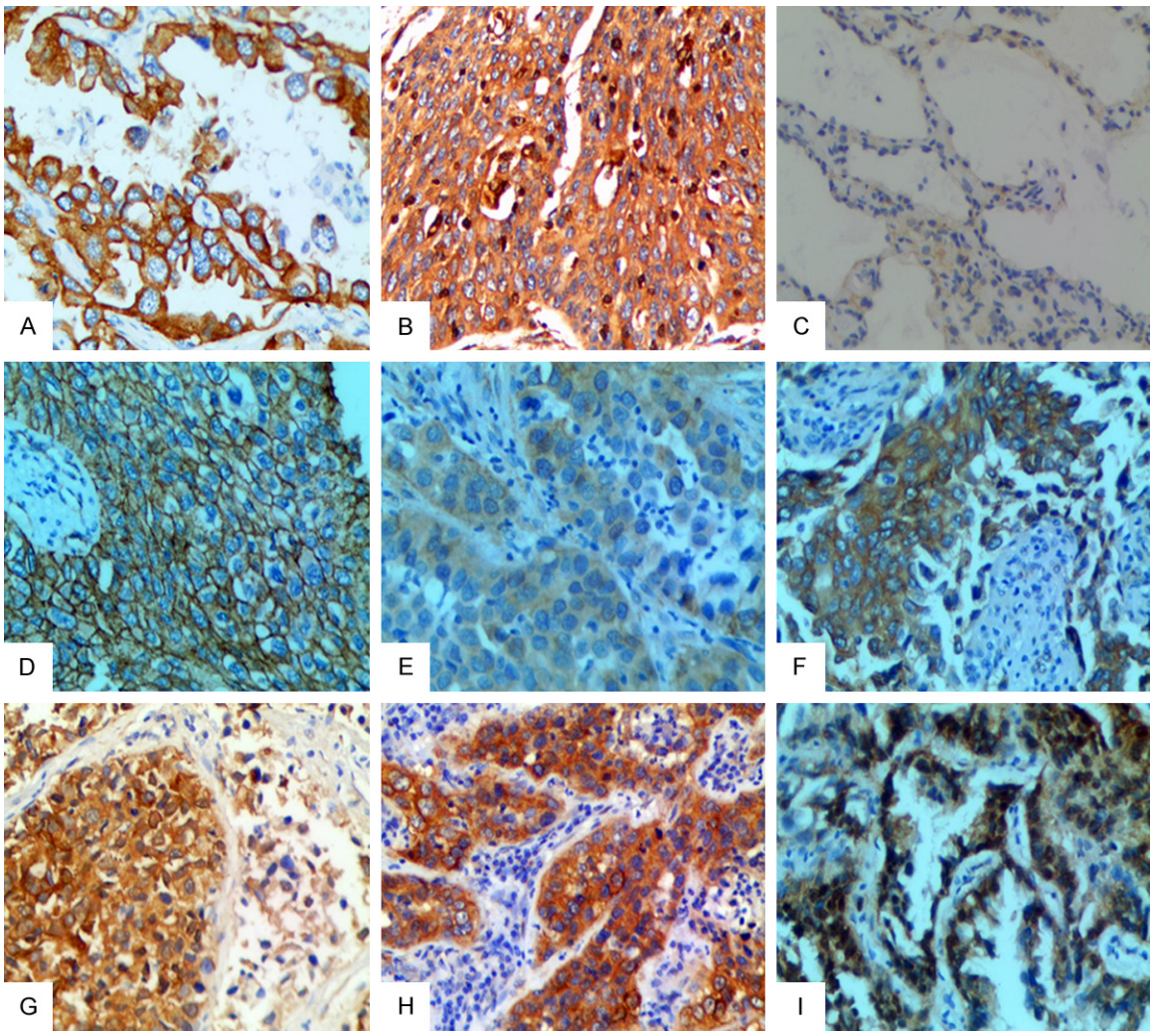

Figure 1. Immunohistochemical expression of DIXDC1 and β-catenin in normal and cancer tissue of NSCLC. A. DIXDC1-positive staining in lung adenocarcinoma. B. DIXDC1-positive staining in lung squamous cell carcinoma. C. DIXDC1-negative staining in normal alveolar epithelial cell. D. Normal membranous pattern of β-catenin. E. The loss of membranous expression of β-catenin and weak staining in cytoplasm. F, G. Membranous-cytoplasmic pattern of β-catenin. H. Strongly cytoplasmic staining and less of membranous staining of β-catenin. I. Cytoplasmic-nuclear pattern of β-catenin. Magnification, 200×.

tions were incubated with DIXDC1 antibody (1:80, ab192776, Abcam company) and β-catenin antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, then followed by incubation with HRP-Conjugated secondary antibodies for 60 min at room temperature, the sections were developed in 0.05% diaminobenziding and counterstained with hematoxylin before dehydration and mounting.

Scoring: The degree of immunoreactivity was evaluated independently by two pathologists without knowledge of the clinical status and outcome data. Protein expression of DIXDC1 is mainly located at the membrane and cytoplasm of the tumor cells. The staining score of DIXDC1 was calculated by staining intensity (on an ordered grade of 0-3: 0, negative; 1, weak; 2, intermediate and 3, strong) × the percentage of positive cells (on an ordered grade of 0-4: 0=0%, 1=1-25%, 2=26-50%, 3=51-75%, 4=76-100%). As the scores for all 20 normal bronchial and alveolar epithelial were <3, a score of 3 was set as the baseline: scores of ≤3 were considered as negative expression (normal expression) and scores >3 as a positive expression (high expression). For β-catenin expression, the staining patterns of β-catenin were divided into four types: (1) a membranous pattern, if immunoreactivity was present solely at the cell mem-

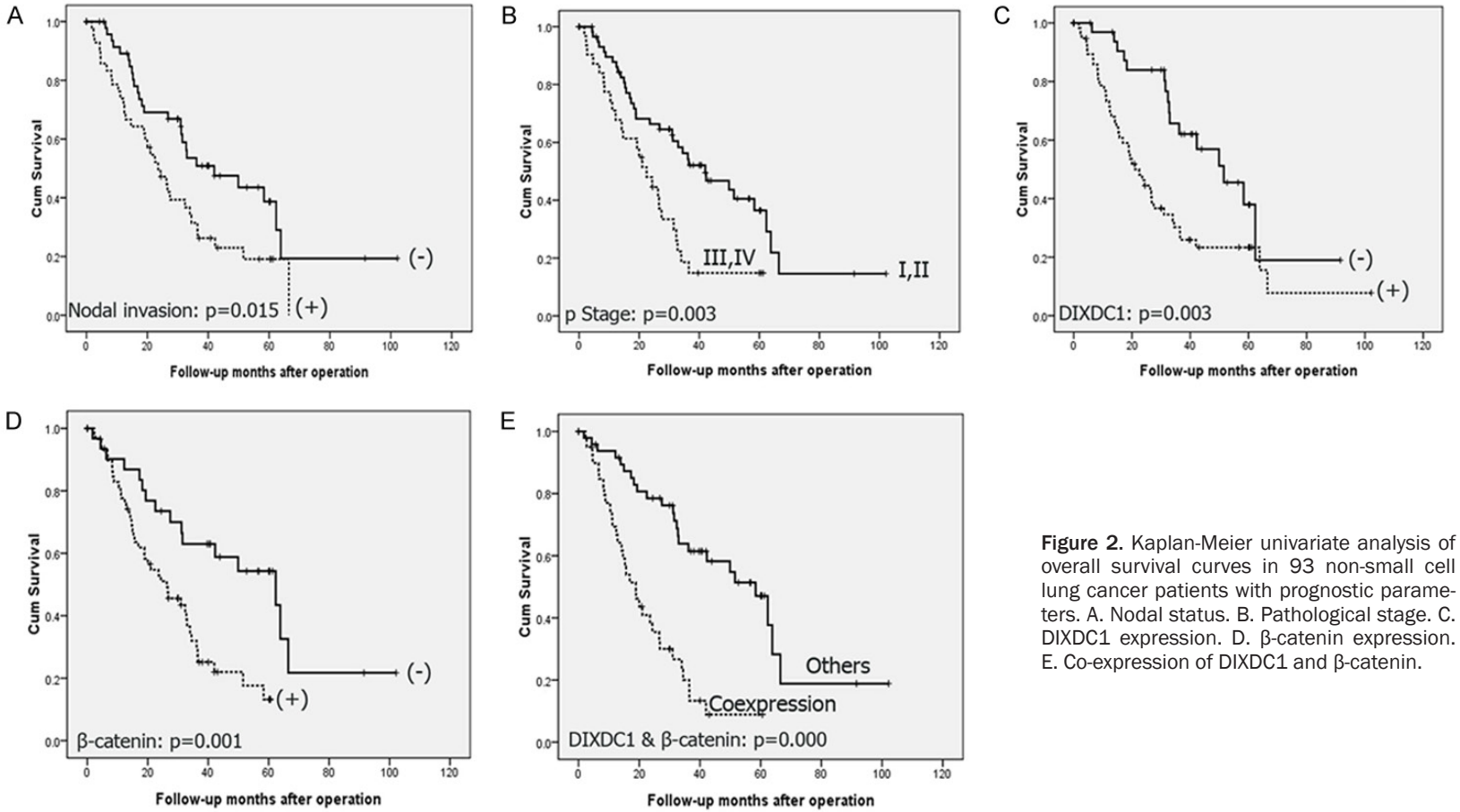

Figure 2. Kaplan-Meier univariate analysis of overall survival curves in 93 non-small cell lung cancer patients with prognostic parameters. A. Nodal status. B. Pathological stage. C. DIXDC1 expression. D. β-catenin expression. E. Co-expression of DIXDC1 and β-catenin.

Table 2. Prognostic significance of tumor variables

Prognostic factor	No	%	Log-rank survival
No. of Patients	93		
Age			
<60 years of age	38	40.9	
≥60 years of age	55	59.1	*P*=0.555
Sex			
Male	65	69.9	
Female	28	30.1	*P*=0.742
Smoking history			
Yes	49	52.7	
No	44	47.3	*P*=0.823
Tumor size			
<5 cm	55	59.1	
≥5 cm	38	40.9	*P*=0.889
Histology			
Squamous	43	46.2	
Adenocarcinoma	50	53.8	*P*=0.774
Grade			
Well/moderately differentiated	77	82.8	
Poorly differentiated	16	17.2	*P*=0.893
T status			
T1, T2	66	71.0	
T3, T4	27	29.0	*P*=0.135
Nodal invasion			
No	49	52.7	
Yes	44	47.3	*P*=0.015*
p Stage			
I, II	59	63.4	
III, IV	34	36.6	*P*=0.003*
DIXDC1			
Negative	35	37.6	
Positive	58	62.4	*P*=0.003*
β-catenin			
Negative	34	36.6	
Positive	59	63.4	*P*=0.001*
DIXDC1 & β-catenin	40	43.0	*P*=0.000*

*represents statistical significance.

brane; (2) a membranous-cytoplasmic pattern, if immunoreactivity was also present in the cytoplasm; (3) a cytoplasmic pattern, if immunoreactivity was chiefly present in the cytoplasm and in less than 20% of the nuclei; and (4) a cytoplasmic-nuclear pattern, if immunoreactivity was present in the cytoplasm and concomitantly in more than 20% of the nuclei. Strongly positive staining specimens of the cytoplasmic-nuclear, membranous-cytoplasmic, and cytoplasmic patterns (≥10%) were taken as positive expression or abnormal expression of β-catenin [14].

Statistical analysis

The analyses were performed using the software package Statistical Package for Social Sciences, version 16.0, for Windows (SPSS, Chicago, IL). The χ^2 test was used to evaluate the association between DIXDC1 and β-catenin proteins and each of the clinicopathologic characteristics. Kaplan-Meier analysis and log-rank test was performed for survival analysis. To evaluate whether a biomarker is an independent prognostic factor of overall survival, multivariate analysis using the Cox proportional hazard regression model was performed. All *p* values were based on the two-sided statistical analysis, and $P<0.05$ was considered as statistically significant difference.

Results

Patient characteristics

Patients consisted of 65 (69.9%) male and 28 (30.1%) female with a median age of 62 years (range, 25-81), with 44 (47.3%) nonsmokers and 49 (52.7%) smokers. The 93 patients were classified using the 7th Edition of the International Union Against Cancer and American Joint Committee on Cancer TNM classification of Malignant Tumors from the International Association for the study of Lung Cancer as follows: Stage I, 15 (16.1%) patients, Stage II, 44 (47.3%) patients, Stage III, 24 (25.8%) patients, Stage IV, 10 (10.8%) patients (**Table 1**). The male to female ratios were 6.2:1 and 1.3:1 in squamous SCC and ADC patients (*P*=0.002). The tumor size of ADC was smaller than that of SCC (*P*=0.002). Among the SCC patients, 65.1% had a history of smoking, but 42.0% of the ADC patients were smokers (*P*=0.026). The age distribution of ADC and SCC had no significant difference (*P*=0.304). The detailed clinicopathologic characteristics in this study are summarized in **Table 1**.

Protein expression of DIXDC1 and β-catenin and association with clinicopathological parameters in NSCLCs

DIXDC1 expression was high in 58 cases of 93 NSCLC, while normal lung tissues exhibited

Table 3. Univariate and multivariate analysis results of overall survival

	Univariate		Multivariate analysis	
Variable	*P* value	Hazard ratio	95% CI	*P* value
Age	0.555	1.022	0.582-1.794	0.939
Grade	0.893	1.150	0.568-2.328	0.697
T1/T2 versus T3/T4	0.135	0.803	0.412-1.568	0.521
Nodal invasion	0.015*	1.118	0.564-2.216	0.750
p Stage	0.003*	1.768	1.015-3.080	0.044*
DIXDC1	0.003*	0.700	0.284-1.729	0.440
β-catenin	0.001*	1.083	0.391-2.998	0.878
DIXDC1 & β-catenin	0.000*	3.408	1.903-6.103	0.000*

*represents statistical significance.

Table 4. Relationship between DIXDC1 expression and β-catenin expression

	DIXDC1 expression		
	Negative	Positive	*p* value
β-catenin			
Negative	18	16	0.021*
Positive	17	42	

*represents statistical significance.

negative or weak staining. This correlated with a positive expression rate of 62.4% (58/93, *P*<0.01, **Figure 1**). Aberrant β-catenin expression was found in 63.4% cases of NSCLC (59/93, *P*<0.01, **Figure 1**). Protein expression of DIXDC1 was significantly positively associated with nodal metastasis and advanced pathological stage (*P*=0.000, *P*=0.001, respectively). Additionally, its expression has marginal effect on T status (*P*=0.05). There is no significant differences between DIXDC1 expression and age, sex distribution, tumor size, smoking history, histotype, grade (*P*>0.05). β-catenin expression was not correlated with age, sex distribution, tumor size, smoking history, T status, histotype, grade, nodal metastasis and pathological stage (*P*>0.05) (**Table 1**).

Prognostic significance of DIXDC1 and β-catenin in NSCLCs

Kaplan-Meier survival analysis showed nodal invasion, pathological stage, DIXDC1, β-catenin expression and co-expression of DIXDC1 and β-catenin indicated as a poor prognosis. The average survival time in nodal metastasis group was 29.19±3.50 months comparing with 48.13±6.06 months of non-metastasis group (*P*=0.015). The average survival time in pathological stage III and IV group is 25.03±3.34 months comparing with 45.84±5.04 months of stage I and II group (*P*=0.003). The average survival time in DIXDC1-positive and -negative groups was 31.85±4.40 and 51.02±6.07 months, respectively (*P*=0.003). The average survival time in β-catenin-positive and -negative groups was 28.78±2.58 and 53.68±7.13 months, respectively (*P*=0.001). The average survival time in co-expression of DIXDC1 and β-catenin group was 22.65±2.67 months comparing with 53.02±5.80 months of other groups, indicating a much poorer prognosis (*P*=0.000) (**Figure 2**). Moreover, age, gender, tumor size, grade, smoking history, histology and T status were not significantly associated with patients' prognosis (**Table 2**). Multivariate Cox regression using the backward elimination method revealed two factors associated with shorter survival. Synchronous overexpression of DIXDC1 and β-catenin (*P*=0.000), higher pathological stage (*P*=0.044) (**Table 3**). In addition, DIXDC1 expression was positively related with aberrant β-catenin expression (*r*=0.240, *P*=0.021) (**Table 4**).

Discussion

Wnt signaling plays a pivotal role in cell proliferation and apoptosis, polarity formation, neural development, and carcinogenesis. DIXDC1 is the human homolog of Ccd1, a DIX domain containing Zebrafish protein, recently identified a positive regulator of the Wnt signaling pathway functioning downstream of Wnt and upstream of Axin [15]. Currently, only three studies have shown that DIXDC1 plays a key role in human cancer. DIXDC1 promotes colon cancer cell growth and increases the invasion and migration ability of non-small cell lung cancer cells via the PI3K/AKT signaling pathway [11, 13]. Wang et al. reported that DIXDC1 activated Wnt signaling pathway and promotes gastric cancer cell invasion and metastasis [12].

In the present study, we investigated prognostic values of DIXDC1 and β-catenin expressions and associations with clinical pathological

parameters in 93 cases of tissue microarray with NSCLC. DIXDC1 protein was mainly localized in the membrane and cytoplasm of tumor cells, its high expression appeared in 62.4% of NSCLC, which was consistent with Xu et al's findings [13], DIXDC1 protein expression was remarkably higher in NSCLC cancer tissues comparing with normal lung tissues. High expression of DIXDC1 in lung cancer was closely associated with lymph node metastasis, pathological stage and poor prognosis. Subcellular localization of β-catenin from cell membrane to the nucleus determines its two distinct functions. As a cell adhesion molecule, membranous β-catenin links E-cadherin to α-catenin. In the nucleus, β-catenin forms a complex with T-cell factor/lymphoid enhancer binding factor to stimulate the expression of Wnt target genes, and played a key role in Wnt/β-catenin signaling pathway. Cytoplasmic and nuclear β-catenin expression has also been reported to be associated with a poor prognosis in patients with cancers of breast, liver, colon and lung [14, 16-18]. In a recent study reported by Li et al. on 309 cases of NSCLC, nuclear β-catenin was strongly associated with poor prognosis of patients with NSCLCs [19]. In agreement with their findings, we showed that aberrant β-catenin expression was significantly associated with poor prognosis of patients with NSCLC. Although multivariate survival analysis by Cox regression model didn't support either DIXDC1 or β-catenin could be an independent prognostic factor, co-expression of DIXDC1 and β-catenin could have an independent prognostic value in NSCLC. The patients with double-positive DIXDC1/β-catenin NSCLC showed significantly shorter survival than other groups.

In addition, the aberrant expression of β-catenin was higher in DIXDC1-positive NSCLCs than in DIXDC1-negative NSCLCs (P=0.021). In the canonical Wnt cascade, β-catenin is the key effector responsible for transduction of the signal to the nucleus and it triggers Wnt-mediated transcription. One study suggests DIXDC1 can stabilize β-catenin by decreasing the phosphorylation level of β-catenin on Ser33/37/41, subsequently increasing its translocation to nucleus in gastric cancer [12]. There is a positive correlation between DIXDC1 and β-catenin expression in our study (r=0.240, P=0.021). Taken together, DIXDC1 might stabilize and promoted β-catenin dependent signaling pathway in progression of NSCLC. Further study need to be clarified the role and mechanism of DIXDC1 in classical Wnt activated signaling pathway in NSCLC.

In conclusion, our data suggest that co-expression of DIXDC1 and β-catenin acts as an independent prognostic factor in NSCLC. DIXDC1 promoting metastasis of NSCLC might be correlated with β-catenin dependent Wnt signaling pathway.

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Disclosure of conflict of interest

None.

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