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Serum Fork-Head Box D3 (*FOXD3*) Expression Is Down-Regulated in and Associated with Diagnosis of Patients with Non-Small Cell Lung Cancer

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: The aim of this study was to detect the expression of fork-head box D3 (*FOXD3*) and investigate its diagnostic value in patients with non-small cell lung cancer (NSCLC).

Material/Methods: The relative expression of *FOXD3* at mRNA and protein levels was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting analysis, respectively. Chi-square test was used to explore the relevance of *FOXD3* expression with clinical features of NSCLC patients. A receiver operating characteristic (ROC) curve was built to estimate the diagnostic value of *FOXD3* in distinguishing NSCLC patients from healthy controls.

Results: Serum *FOXD3* expression was weakly expressed in NSCLC patients compared to the controls at mRNA and protein levels ($P<0.001$) and low *FOXD3* expression was positively correlated with TNM stage, lymph node metastasis, and differentiation. The ROC curve indicated that *FOXD3* acts as a diagnostic bio-marker for NSCLC patients, with an AUC of 0.826 corresponding to a sensitivity of 77.1% and a specificity of 74.6%, and an optimal cutoff point of 2.38.

Conclusions: Decreased expression of serum *FOXD3* was observed in NSCLC patients, and it was found to be a potential molecular marker for the diagnosis of NSCLC.

MeSH Keywords: Carcinoma, Non-Small-Cell Lung • Diagnosis • Forkhead Transcription Factors

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Background

Lung cancer, the main cause of cancer-related deaths, is the most common malignancy all over the world [1]. NSCLC is a common disease and accounts for about 80% of all lung cancers [2,3]. Currently, the main treatments for NSCLC patients are surgical resection, radiation therapy, and chemotherapy [4,5]. Although remarkable improvements have been made in those treatments, the curative effects are still unsatisfactory and over 160 000 patients die per year worldwide [6,7]. Because most NSCLC patients are diagnosed at late stage, the prognosis of NSCLC patients is poor and the 5-year survival rate for NSCLC patients is less than 15% [8–10]. Therefore, novel and available bio-markers for the diagnosis of NSCLC patients are urgently need.

Fork-head box D3 (*FOXD3*) is a protein-coding gene that locates at chromosome 1p 31.3 and a winged-helix transcription factor that belongs to the fork-head box (FOX) transcription factor family [11–13]. It was initially identified because of its expression in embryonic stem cells, and it plays multiple important roles in vertebrate embryogenesis, including control of dorsal mesoderm formation, maintenance of self-renewal and multipotency of stem cells, and regulation of neural crest development [14–18]. Ectopic expression of *FOXD3* has been proved to suppress the invasion, migration, and spheroid outgrowth of mutant B-RAF melanoma cells [19]. In addition, dysregulation of *FOXD3* has been observed in gastric cancer, breast cancer, and neuroblastoma [20–22]. However, its diagnostic value in NSCLC has rarely been reported.

In the present study, we investigated the expression of *FOXD3* and analyzed the correlation of *FOXD3* expression and clinical features. We also assessed the diagnostic role of *FOXD3* in NSCLC.

Material and Methods

Patients

The present study was performed in the Fourth Affiliated Hospital of China Medical University and was approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University during April 2011 and June 2015. A total of 131 patients who were diagnosed with NSCLC were enrolled in the present study. None of the patients had received any preoperative treatment, including radiotherapy and chemotherapy, before sampling. In addition, 63 healthy volunteers were enrolled as healthy controls. Written informed consent was provided by each participant in advance.

We obtained 10 ml peripheral blood from each participant and stored the samples at room temperature. After centrifugation at 3000 rpm at 4°C, the obtained serum was put into EDTA blood collection tubes and stored at –80°C until use. Clinicopathologic characteristics of each patient were recorded in a database.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from serum samples using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Then, the first chain of cDNA was synthesized via reverse transcription using the Primer Script RT Master Kit (TAKARA, China). RT-PCR reaction was performed using the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, USA). U6 small nuclear (U6) was used as internal control. The relative mRNA expression of *FOXD3* was calculated using the $2^{-\Delta\Delta t}$ method. Each sample was assessed in triplicate.

Western blotting

Total protein was isolated from all samples. The protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis (SDS-PAGE). The brands were transferred onto nitrocellulose filter membranes. After the membranes were blocked with 5% non-fat milk, they were incubated with primary antibodies against FOXD3 or GPADH (Abcam, Cambridge, MA, USA), followed by incubation with horseradish peroxidase-conjugated IgGs. Target protein was detected by an enhanced chemiluminescence substrate kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

The data were analyzed using SPSS 18.0 software and the figures were designed with GraphPad Prism 5. The differences between 2 groups were analyzed by *t* test. The chi-square test was used to analyze the relationship of *FOXD3* expression with clinical parameters of NSCLC patients. The ROC curve was plotted to evaluate the diagnostic value of *FOXD3* in patients with NSCLC compared to healthy controls. The difference was considered to be statistically significant when *P* was less than 0.05.

Results

Down-regulation of serum *FOXD3* at mRNA level was observed in NSCLC patients

The relative mRNA expression of serum *FOXD3* in NSCLC patients and healthy controls was determined by qRT-PCR analysis. As shown in Figure 1, the serum *FOXD3* expression in NSCLC

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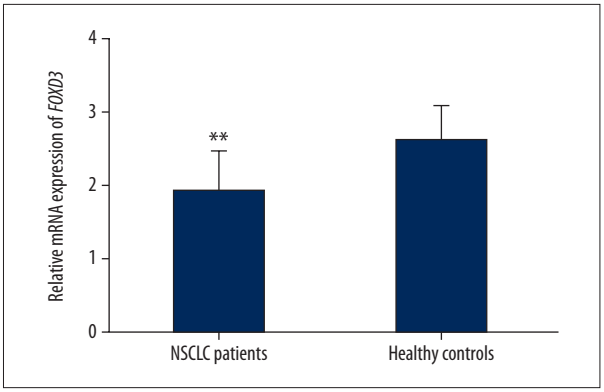

Figure 1. The relative mRNA expression of serum *FOXD3* in NSCLC patients and healthy controls. The serum *FOXD3* expression was lower in NSCLC patients than that in healthy controls (*P*<0.001).

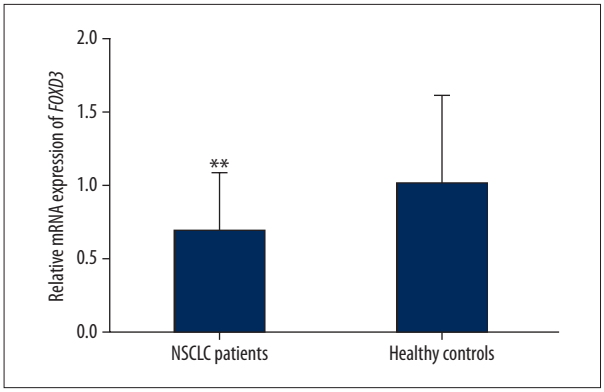

Figure 2. The relative protein expression of serum *FOXD3* in NSCLC patients and healthy controls. The serum *FOXD3* protein expression was decreased in NSCLC patients compare to that in healthy controls (*P*<0.001).

Table 1. Relationship between *FOXD3* expression and clinical parameters of patients with NSCLC.

Clinical parameters	Case (n=131)	*FOXD3* expression		χ^2	*P* value
		Low (n=94)	High (n=37)		
Age				0.090	0.764
≤55	61	43	18		
>55	70	51	19		
Histology				1.863	0.172
Squamous cell carcinoma	69	46	23		
Adenocarcinoma	62	48	14		
Smoking				0.332	0.564
Yes	76	56	20		
No	55	38	17		
TNM stage				6.092	0.014
I, II	66	41	25		
III, IV	65	53	12		
Lymph node metastasis				5.336	0.021
Yes	74	59	15		
No	57	35	22		
Differentiation				4.421	0.035
High/moderate	58	47	11		
Low	73	47	26		

patients was lower than that in healthy controls (2.01±0.44 versus 2.62±0.46, *P*<0.001).

The protein expression of *FOXD3* in NSCLC patients

Western blotting was used to measure the protein expression of *FOXD3* in NSCLC patients and healthy controls. The result demonstrated that serum FOXD3 protein was decreased in NSCLC patients compared to healthy controls (0.69±0.39 *vs.* 1.04±0.59, Figure 2, *P*<0.001).

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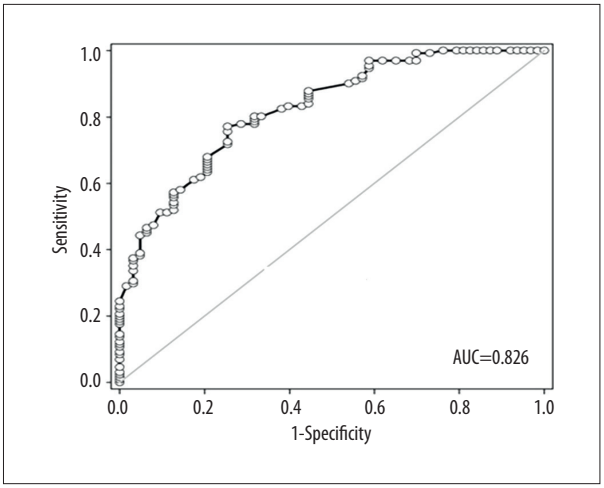

Figure 3. The diagnostic value of *FOXD3* in patients with NSCLC was analyzed by establishing an ROC curve. The cutoff point was 2.38 and the AUC was 0.826 (*P*<0.001, 95%CI=0.766-0.885).

Association of *FOXD3* expression and clinical parameters of NSCLC patients

To analyze the relationship of *FOXD3* and clinical features of NSCLC patients, the chi-square test was used. The outcome revealed that the low expression of *FOXD3* was positively associated with TNM stage (*P*=0.014), lymph node metastasis (*P*=0.021), and differentiation (*P*=0.035). However, there was no significant difference between *FOXD3* expression and age, histology, or smoking (Table 1, *P*>0.05).

The diagnostic value of *FOXD3* in NSCLC

The ROC curve was plotted to assess the diagnostic potential of *FOXD3* in patients with NSCLC. The outcome demonstrated that *FOXD3* expression was able to efficiently distinguish NSCLC patients from controls (AUC=0.826, 95%CI=0.766-0.885, *P*<0.0001). According to the ROC curve (Figure 3), the optimal cutoff point of *FOXD3* expression was 2.38. Based on the cutoff point, a sensitivity of 77.1% and a specificity of 74.6% were obtained.

Discussion

NSCLC is a common, slow-developing lung cancer with complex pathogenesis; its progression involves in not only several stages, but also many oncogenes and tumor suppressor genes [23,24]. Bai et al. found that *miR-32* was associated with progression and predicted the prognosis of NSCLC [25]. Jiang et al. [26] reported that *HE4* serves as a tumor marker for NSCLC, and its low expression can predict a poor prognosis of NSCLC patients. Ke et al. revealed that the over-expression of *CTHRC1* was related to tumor aggressiveness and prognosis of human NSCLC [27]. It was demonstrated by Ji et al. that low expression of *PTRN* and over-expression of *Ki67* were correlated with lymph node metastasis and malignant invasion of NSCLC [23].

FOXD3 is a member of the winged-helix/fork-head transcription factor family, which is characterized by a conserved domain containing 100 residues and that is required for the activity of DNA-binding [18]. It is mainly expressed in the multi-potent cells, including embryonic stem cells and tumor cells. Reports have revealed that *FOXD3* plays important roles in the maintenance and self-renewal of embryonic stem cells, and is strongly related to the early development of embryos [23]. Studies on the effects of *FOXD3* on tumor stem cells have also been reported. Steiner et al. suggested that *FOXD3* was essential for the development of xenopus dorsal mesoderm [28]. It was reported by Teng et al. that *FOXD3* was required in maintaining the progenitors of the neural crest [29]. All these reports provide theories relevant to studying the relationship between *FOXD3* and NSCLC.

In addition, Ectopic expression of *FOXD3* has been reported in various diseases. Wang et al. reported that *FOXD3* was down-regulated in lung cancer and could suppress the development of this disease [11]. Low expression of *FOXD3* in breast cancer was reported by Zhao et al. [21]. In the present study, we first detected the expression of *FOXD3* in NSCLC patients, showing that the expression level of *FOXD3* was significantly lower in NSCLC patients than in controls at mRNA and protein levels, which was in accord with previous studies. It can be concluded that *FOXD3* might be a tumor suppressor in NSCLC.

Then, we assessed the correlation of *FOXD3* with the tumorigenesis of NSCLC by analyzing the relationship of *FOXD3* expression and clinical features of NSCLC patients. *FOXD3* was proved to have close relationships with differentiation, TNM stage, and lymph node metastasis. Subsequently, in order to explore the diagnostic value of *FOXD3* in patients with NSCLC, an ROC curve was built. As a result, a high AUC, as well as high sensitivity and specificity, were obtained, showing that *FOXD3* is an independent diagnostic marker in NSCLC.

In previous studies, the precise mechanism and function of *FOXD3* in the tumorigenesis and progression of diseases were revealed and it was reported that it cannot be regulated by some genes. For instance, Basile et al. showed that *FOXD3* affects mutant B-RAF melanoma cells via regulating *PLX4032*/4720 [30]. Guo et al. found that *FOXD3* interacted with *Oct-4* to regulate the expression of endodermal specific promoter [31]. Liu et al. demonstrated that *FOXD3* regulates the expression of microRNA-137 tumor growth in hepatocellular cancer [32]. However, its mechanisms in NSCLC remains unclear. Our group plans to explore this topic further in future research

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Conclusions

FOXD3 is down-regulated and plays an important role in the development of NSCLC. In addition, it can act as a diagnostic bio-marker for patients with NSCLC. The present study is limited by its small samples size and further studies are needed.

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