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Original Article

Wnt2 promotes non-small cell lung cancer progression by activating WNT/β-catenin pathway

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Abstract: Background: Wnt2 is overexpressed and able to promote tumorigenesis in many types of cancer. However, its expression and role in lung cancer has not been well clarified yet. In this study, we aims to investigate the expression pattern, clinical significance and the underlying molecular mechanism of Wnt2 in non-small lung cancer (NSCLC). Methods: Immunohistochemical staining and ELISA assays were applied to detect Wnt2 level in tumor tissue and serum. EDU incorporation assays and colony formation assays were used to evaluate the growth-promoting effect of Wnt2 in vitro. Then we performed western blot and immunofluorescence assays to detect the activation of WNT signaling pathway. Finally mice engrafted with NSCLC tumor cells were used to assess the role of Wnt2 in vivo. Results: Immunohistochemical staining consisting of 264 NSCLC tumor tissues showed that a high level of Wnt2 was associated with a poor overall survival (OS) and relapse-free survival (RFS) of NSCLC patients (P = 0.002 and 0.0005, respectively). Multivariate analysis presented that Wnt2 level in tumor tissue was an independent prognostic factor (P = 0.049 for OS and P = 0.002 for RFS, respectively). Furthermore, ELISA assays for 181 individuals (116 NSCLC and 65 controls) revealed that serum Wnt2 levels in adenocarcinoma was significantly higher than that in healthy volunteers (P < 0.0001). In vitro H460 cell line stably overexpressing Wnt2 showed enhanced growth activity than the control cells whereas knockdown of Wnt2 by siRNA in H1299 cells resulted in decreased growth activity. Additionally, Wnt2 level in tumor tissues was significantly associated with Ki-67 level (rs: 0.316; P < 0.0001). Immunofluorescence and Western blot assays detected the translocation of β-catenin from cytoplasm into nucleus, which indicated that Wnt2 probably promotes proliferation by activating WNT/β-catenin pathway. In vivo H460 cells expressing exogenous Wnt2 showed increased growth-promoting effect in Balb/c nude mice than control cells. Conclusions: The present study for the first time suggested that Wnt2 was both a prognostic and a diagnostic biomarker for NSCLC. Tumor-derived Wnt2 can promote growth activity of NSCLC cells through activating WNT/β-catenin signaling pathway.

Keywords: Wnt2, WNT signaling pathway, non-small cell lung cancer, proliferation, tumor biomarker, β-catenin

Introduction

Lung cancer is one of the leading causes of global malignancy-related mortality, resulting in over one million deaths each year. Non-small cell lung carcinoma (NSCLC) accounts for approximately 85% of lung cancers [1]. Despite the different therapeutic strategies developed to date, the 5-year survival rate of patients with NSCLC is only 10% to 15%. However, more than 80% patients with NSCLC staged IA can survive over five years [2]. A serial of tumor markers such as squamous cell antigen (SCC), carcinoembryonic antigen (CEA), and serum cytokeratin 19 fragment (CYFRA21-1) are used to diagnose lung cancer, however, these markers are not perfect enough to detect cancers in early-stage because of relatively low sensitivity and specificity [3]. The ability to predict the prognosis of patients would help them and their doctors selecting appropriate treatment approaches. Additionally, prognostic biomarkers may shed some light on novel therapeutic targets. Although several prognostic factors had been

reported, tumor pathological stage and performance status are still the most significant factors. As a result, the development of novel diagnostic and prognostic tumor markers is necessary.

The WNT signaling pathway is crucial in the regulation of cell proliferation, differentiation, apoptosis and migration. Abnormal expression and activation of WNT signaling pathway can induce tumors [4, 5]. Wnt proteins are a family of secreted glycoproteins and signal through the frizzled (Fz) receptors in a paracrine pattern [6]. The Wnt signaling pathways could be classified into three major types. The most known one is the canonical pathway, which activates downstream genes through inhibition of GSK-3activity, followed by accumulation of β-catenin in nucleus. Other Wnt signaling pathways include the Wnt/Ca^{2+} pathway, which activates calmodulin kinase II and protein kinase C, and the planar cell polarity pathway (PCP), which features an increase of Jun N-terminal kinase [7]. WNT2 gene located on human chromosome 7q31 is a paralog of the WNT2B gene on human chromosome 1p13. Many researchers have revealed its involvement in tumorigenesis and development in varied types of tumors through activation of the canonical pathway, such as oesophageal cancer [8], gastric cancer [9], colorectal cancer [10], breast cancer [11] and pancreatic carcinoma [12].

A few studies had reported the expression of Wnt2 in NSCLC. Bravo DT [13] et al. reported that Wnt-2 signaling was activated in NSCLC, using a semi-quantitative RT-PCR method. Liang You [14] et al. revealed that Wnt2 protein was overexpressed in fresh human NSCLC tissues and could induces programmed cell death in vitro. However, none of the aforementioned studies had described the expression level of Wnt2 protein in NSCLC tumor tissue on a large clinical sample, as well as that in NSCLC serum samples. Additionally, the clinical significance of Wnt2 expression in NSCLC as well as the underlying mechanism had not been elucidated. We here retrospectively probed the expression pattern of Wnt2 both in 264 tumor tissues and 181 serum samples and then explored the clinical significance. Subsequently we studied the effect of Wnt2 in tumor progression both in vitro and in vivo as well as the underlying mechanism.

Materials and methods

Cell lines, patient selection and sample collection

All the five NSCLC cell lines and 293T cell line used in this study were bought from the Cell Culture Center of the Shanghai Institute for Biological Sciences (Chinese Academy of Science, Shanghai, China). Three adenocarcinoma cell lines were A549, H1299 and H1975. One squamous cell line was SK-MES-1. One large cell carcinoma cell line was H460. The cancer cells were grown in monolayer in 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in humidified air with 5% CO_2.

A total of 264 formalin-fixed and paraffin-embedded NSCLC tissues and adjacent normal lung tissues were collected earlier in Tianjin Medical University Cancer Hospital and Institute (TJMUCH). Retrospective clinicopathological data of these patients were also obtained, including age, gender, tumor size, regional lymph node status, TNM stage, pathologic type and differentiation. The patient background was summarized in Supplementary Table 1.

Serum samples were also collected earlier in TJMUCH with informed consent from 181 individuals comprising 65 healthy volunteers and 116 NSCLC patients. Blood samples were obtained at the time of diagnosis, centrifuged at 300g for 15 minutes and then the serum was stored at -80°C.

This study was approved by the Ethics Committee of TJMUCH. All patients signed a written consent for the use of their specimens and disease information for future investigations according to the ethics committee.

siRNA interference, transient transfection plasmid construction and stable transfection cell lines

siRNAs against Wnt2 were designed and synthesized by Genepharma. The target sequences for RNA interference were as follows: SiRNA-1, 5'-GCGCAUUUGUGGAUGCAAATT-3'; SiRNA-2, 5'-GAAGATGGGAAGCGCCAAG-3'. A scramble siRNA sequence (5'-UUCUCCGAACGUGUCACGUTT-3') was used as a control. For transfec-

tion, cells were plated at a density of 5×10^5 cells/well in 6-well plates. When the cells reached 80% confluent, 10 n mol siRNAs were transfected into cells using Lipofectamine 3000 (Invitrogen) for 48 hours according to the manufacturer's instructions.

The human Wnt2 gene was amplified by PCR and then cloned into pLV-EF1-MCS-IRES-Bsd vector (Biosettia). Lentiviruses were produced in 293T cells for stable transfection of cell lines, following the manufacturer's instructions. Empty vector was transfected into cells to be used as control. Briefly, seed 6.0×10^6 293T cells in a 10 cm plate and then transfected with pLV-cDNA together with VSV-G, Rev, and Gag-Pol vectors by Lipofectamine 3000 (Invitrogen). After 16 h, the transfection solutions were replaced with complete medium. The culture supernatants containing lentiviruses were collected at 48 h. A total of 1×10^5 H1299 cells in 2 ml medium with 8 μg/ml polybrene were infected with 1 ml lentivirus supernatant. 48 h later blasticidin (InvivoGen) was added for selection.

Semi-quantitative RT-PCR

Eight pairs of NSCLC samples and adjacent normal lung tissues were grinded in liquid nitrogen. Total RNA of cells and the tissues was extracted by Trizol (Invitrogen) according to the manufacturer's instructions. Then a total of 3 mg mRNA was reversely transcribed to single-stranded cDNAs by using a reverse-transcription PCR (RT-PCR) system (TaKaRa). β-actin (ACTB) was used as a loading control. The primers for Wnt2 and ACTB were Wnt2 forward, ATGTCACCCGGATGACCAAG; Wnt2 reverse, 5'-TCCAGAGCTTCCAGGCAGTC-3'; ACTB forward, 5'-CTACCTTCAACTCCATCATGAAGTG-3'; ACTB reverse, 5'-TGCGCTCAGGAGGAGC-3'. The number of cycles was optimized to ensure that product intensity was within the linear phase of amplification. Products of semi-quantitative PCR were detected by agarose gel electrophoresis.

Immunohistochemical staining

Immunohistochemistry for Wnt2 and Ki67 of NSCLC patient tissues was performed according to the manufacturer's instructions. In brief, paraffin-embedded sections of NSCLC were deparaffinized and then heated in a pressure pot for 3 minutes to retrieve antigens. Then the sections were incubated with rabbit anti-human Wnt2 monoclonal antibody at 1:250 dilution (Abcam) or rabbit anti-human Ki67 polyclonal antibody at 1:200 (BOSTER) overnight at 4°C. The slides were then incubated with peroxidased goat anti-rabbit/mouse secondary antibody (Maxin) at 37°C for 30 min. A DAB Substrate Kit (Maxin) was used to do the chromogenic reaction. The results were scored by two experienced pathologic examiners who were unknown of the clinicopathologic data. The intensity of Wnt2 staining was evaluated by using the following criteria: 0, negative; 1, low; 2, medium; 3, high. Extent of staining was scored as 0, 0% stained; 1, 1% to 25% stained; 2, 26% to 50% stained; 3, 51% to 100% stained. Five random fields (20 × in magnification) were evaluated under a light microscope. The final scores were calculated by multiplying the scores of intensity with that of extent. The staining results were divided into four grades by final scores: 0, negative (-); 1 to 2, low staining (+); 3 to 4, medium staining (++); 6 to 9, high staining (+++). The Ki67 labelling index was used to evaluate the staining Ki67 as described previously [15].

Immunofluorescence

Cells cultured with Wnt2 recombinant protein (rWnt2, Abnova) or IgG control (Sino Biological Inc.) at a concentration of 200 ng/mL were seeded on coverslips for the indicated time points and then fixed in 4% paraformaldehyde at room temperature for 15 min. Cells were incubated with 3% bovine serum albumin in PBS solution at room temperature for 60 min to block nonspecific interactions. The coverslips were added with rabbit anti-human β-catenin monoclonal antibody (Cell Signaling Technology) and incubated at 4°C overnight. After washes in PBS, cells were incubated with fluorescent dye-labeled goat anti-rabbit IgG secondary antibodies (Life Technologies) at room temperature for 1 h. Anti-fade DAPI solution (1:1000) was added and images were obtained by a fluorescence microscope.

Western blotting analysis

Eight pairs of NSCLC samples and adjacent normal lung tissues were grinded in SDS lysis buffer. Whole-cell extracts were prepared by lysing cells with SDS lysis buffer supplemented with

proteinase inhibitors cocktail (Sigma). A nuclear and cytoplasmic protein extraction kit (Thermo) was used to separate nuclear and cytoplasmic protein, following the manufacturer's instructions. A total of 20 mg protein lysates were separated by SDS-PAGE and then the target proteins were detected by Western blot analysis with the following antibodies: rabbit anti-human Wnt2 monoclonal antibody (Abcam), rabbit anti-human β-catenin monoclonal antibody (Cell signaling Technology) and mouse anti-human β-actin monoclonal antibody (Abmart).

ELISA

A human Wnt2 ELISA kit (CUSABIO) was used to detect Wnt2 concentration in the serum samples or the supernatants of cell cultures.

Colony formation assay and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

Colony formation assay was carried out as described previously [16]. In brief, six-hole plate was seeded at a density of 800 cells per hole. Plates were maintained at 37°C in humidified incubator and culture medium was replaced every 3 days. After 2 weeks, cells were stained with 0.5% Crystal Violet and the average colony number of five random fields (4 × in magnification) was counted under microscope. A EDU incorporation kit (Ribobio) was used to measure the DNA synthesis rate as described [17]. All experiments were conducted in triplicates. Representative views were photographed.

In vivo assay

H1299 cells transfected with Wnt2 expression vector or empty vector were suspended in phosphate buffer solution. Then the cell suspension were injected subcutaneously into the inguinal region of 6 pairs of 4-week-old male Balb/c nude mice (Vital River Laboratory Animal Technology Co. Ltd, China). Tumor diameter was measured every week until the mice were anesthetized and sacrificed at the end of week 6. Tumor volume was determined by the longest and shortest diameter of the tumor and calculated as follows: volume = (shortest diameter)^2 × (longest diameter) × 0.5 [18]. The animal handling and all experimental procedures were approved by the Animal Ethics Committee of TJMUCH.

Statistical analysis

Statistical analyses were carried out by the IBM SPSS Statistics Program. Each experiment was done in triplicate and values are presented as mean ± SD. A Student t test or ANOVA for unpaired data was used to compare mean values. Kaplan-Meier curves were calculated for relevant variables and Wnt2 expression; The log-rank test was used to analyze the differences in survival times among patient subgroups. The risk factors associated with the prognosis of these patients were evaluated by Cox's proportional hazard regression model. The Forward: LR procedure was used for the univariate analysis. All probability values had a statistical power level of 90% and a 2-sided level of 5%. Serum Wnt2 levels before and after surgery were analyzed by the paired t test. Receiver operating characteristic (ROC) curve analysis was performed in MedCalc Program to compare Wnt2 and CEA for their role in predicting NSCLC. $P < 0.05$ was considered significant.

Results

Wnt2 expression in NSCLC samples, adjacent normal tissues and NSCLC cell lines

To explore the role of Wnt2 in NSCLC progression, we detected Wnt2 expression level in 8 pairs of NSCLC samples and adjacent normal lung tissues by semiquantitative RT-PCR and Western blot assay (**Figure 1A**). As shown in **Figure 1B**, the transcription and translation level of Wnt2 in cancer samples was obviously higher than in corresponding adjacent normal lung tissues. Among them, 4 pairs belonged to patients with adenocarcinoma (ADC) while the other half came from patients with squamous cell lung cancer (SCC). Intriguingly, Wnt2 expression in ADC was obviously higher than that in SCC. To further understand the clinicopathologic significance of Wnt2 in NSCLC samples, we subsequently used immunohistochemistry to determine Wnt2 levels in a cohort of 264 patients with NSCLC treated in TJMUCH. We classified a pattern of Wnt2 expression ranging from absent (scored as 0) to low, median and high positive (scored as 1+, 2+ to 3+; **Figure 1D**). Wnt2 was positively stained in the majority of NSCLC tissues but not in their corresponding adjacent normal lung tissues. Wnt2 expression could be detected in majority types of NSCLC

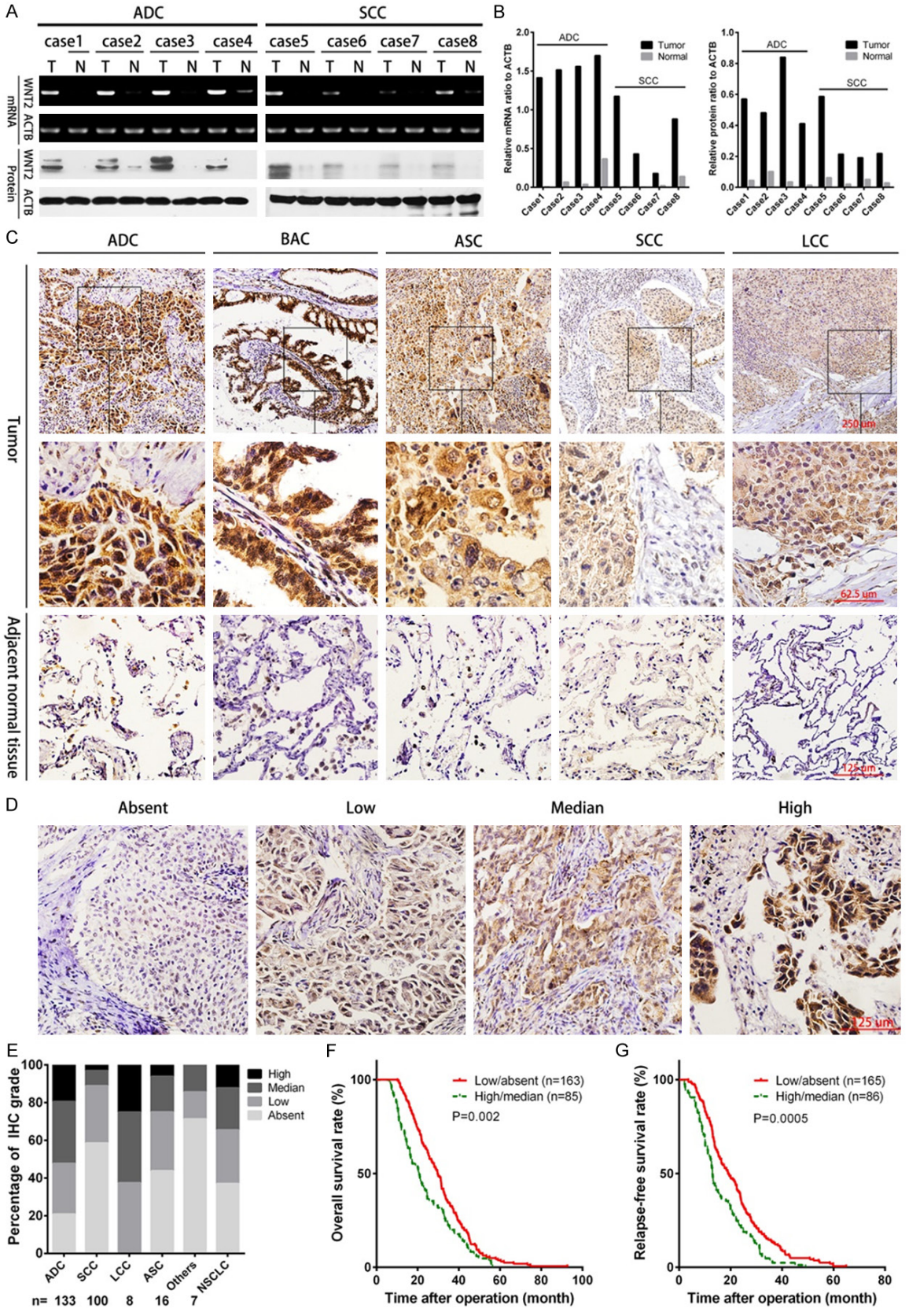


Figure 1. Expression of Wnt2 in normal tissue and non-small cell lung cancers. (A) expression of Wnt2 in 8 representative pairs of NSCLC lung cancer tissues (T) and adjacent normal lung (N) tissues detected by RT-PCR (top) and Western blot (bottom). The results were processed with ImageJ (B). (C) immunohistochemical staining of Wnt2 pro-

tein in 5 representative pairs of histologic types of NSCLC (ADC, BAC, ASC, SCC and LCC) and adjacent normal lung tissues. (D) examples are shown for absent, low, median and high Wnt2 expression in NSCLC cancer tissues. (E) distribution of Wnt2 immunohistochemical results in 264 NSCLC patients as well as that according to pathological subtypes (ADC, SCC, LCC, ASC and others). Kaplan–Meier analysis of overall survival (F) and relapse-free survival (G) of patients with NSCLC (P = 0.002 by log-rank test) according to expression of Wnt2 protein. Vertical bars on survival curves indicate censored cases.

Table 1. Association between WNT2 IHC staining and patients' clinicopathological parameters (n = 264)

	Total (n = 264)	High/median expression (n = 91)	Low/absent expression (n = 173)	x^2	*P*
Gender					
Male	163	58	105	234	0.629
Female	101	33	68		
Age,y					
< 65	171	62	109	0.687	0.407
≥ 65	93	29	64		
Pathologic type					
ADC	134	68	65	32.927	< 0.0001
Non-ADC	130	23	108		
pT stage					
T1+T2	229	70	159	11.643	0.001
T3+T4	35	21	14		
pN stage					
N0	28	6	22	2.358	0.125
N1+N2	236	85	151		
Differentiation					
Well+Moderate	177	58	119	0.688	0.407
Poor	87	33	54		

P values were calculated by chi square test. ADC, adenocarcinoma.

(**Figure 1C**). The number of NSCLC tissues scored as 0, 1, 2 and 3 was 98 (37.1%), 75 (28.4%), 59 (22.3%) and 32 (12.1%), respectively (**Figure 1E**). As shown in **Table 1**, ADC pathotype ($P < 0.0001$) and advanced pathologic tumor stage ($P = 0.001$) were significantly associated with high Wnt2 positivity. Kaplan-Meier curve analysis was performed. As shown in **Figure 1F** and **1G**, the OS and RFS of patients with NSCLC showing low/absent Wnt2 staining was significantly longer than that of patients with high/median Wnt2 staining ($P = 0.002$ and 0.0005 respectively). The median OS for absent/low Wnt2 group and high/median group were 29.58 months and 20.62 months respectively, whereas the median RFS were 20.03 months and 13.16 months respectively. To evaluate the prognostic significance of the clinicopathological features and Wnt2 in patients with NSCLC, we conducted Cox's proportional hazard regression analysis on the parameters listed in **Table 2**. Multivariate analysis revealed that Wnt2 expression ($P = 0.049$ and 0.002, respectively) as well as pathologic tumor stage, pathologic node stage and differentiation were independent prognostic factors for OS and RFS of patients with NSCLC who received surgery treatment.

Subsequently we detected the level of Wnt2 in 5 NSCLC cell lines. Unsurprisingly, the results of RT-PCR, WB and ELISA assays showed that Wnt2 was abnormally expressed in these cells (**Figure 2**).

Serum levels of Wnt2 in patients with NSCLC

Because Wnt2 was a secreted protein, we performed ELISA assays to evaluate whether Wnt2 protein could be used as a serologic tumor marker for NSCLC. We detected Wnt2 serum level in samples obtained from 116 NSCLC patients and 65 healthy volunteers. The mean serum Wnt2 levels in NSCLC patients and healthy volunteers were 5.68 ± 6.03 and 2.47 ± 2.11 ng/mL, respectively. The serum levels of Wnt2 were significantly higher in patients with NSCLC than in healthy volunteers ($P < 0.0001$). As shown in **Figure 3A**, when classified according to pathologic type, the serum Wnt2 levels in healthy volunteers, ADC patients and non-ADC patients were 2.47 ± 2.11 ng/mL, 8.87 ± 7.06 ng/mL and 2.81 ± 2.68 ng/mL respectively. The serum Wnt2 levels in ADC patients were significantly higher than that in healthy donors or in non-ADC patients ($P < 0.0001$). Compared to healthy volunteers, non-ADC patients did not had a significant elevation in serum Wnt2 levels

Table 2. Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

Variables	Unfavorable/favorable	Overall survival (OS)		Relapse free survival (RFS)	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Univariate analysis					
WNT2	Strong (+)/weak (+)or (-)	1.485 (1.080-2.042)	0.015	1.618 (1.198-2.185)	0.002
Gender	Female/male	0.732 (0.803-1.366)	0.732	0.990 (0.765-1.280)	0.937
Age	≥ 65/< 65	0.935 (0.706-1.239)	0.642	0.870 (0.663-1.140)	0.312
Pathologic type	Non-ADC/ADC	1.250 (0.933-1.675)	0.012	1.321 (1.001-1.742)	0.049
pT stage	(T3+T4)/(T1+T2)	3.160 (2.108-4.737)	< 0.0001	2.320 (1.596-3.373)	< 0.0001
pN stage	(N1+N2)/N0	3.437 (2.154-5.486)	< 0.0001	3.025 (1.950-4.692)	< 0.0001
Differentiation	Poor/(moderate+well)	1.594 (1.208-2.103)	0.001	1.341 (1.028-1.750)	0.031
Multivariate analysis					
WNT2	Strong (+)/weak (+) or (-)	1.319 (1.001-1.739)	0.049	1.589 (1.180-2.141)	0.002
pT stage	(T3+T4)/(T1+T2)	3.351 (2.273-4.939)	< 0.0001	2.302 (1.584-3.346)	< 0.0001
pN stage	(N1+N2)/N0	3.446 (2.183-5.442)	< 0.0001	2.960 (1.912-4.582)	< 0.0001
Differentiation	Poor/(moderate+well)	1.582 (1.211-2.066)	0.001	1.316 (1.012-1.712)	0.040
Pathologic type	Non-ADC/ADC	1.310 (0.995-1.726)	0.055	-	-

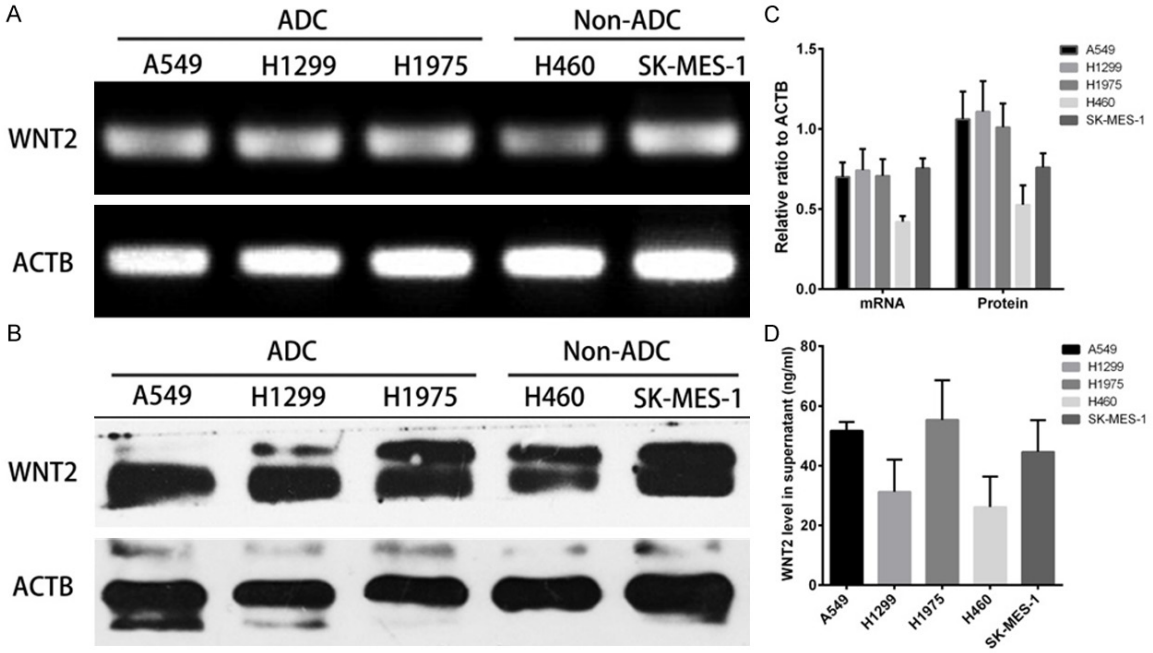


Figure 2. Expression of Wnt2 expression in 5 NSCLC cell lines. Expression of Wnt2 in 5 NSCLC cell lines (3 adenocarcinoma, A549, H1299 and H1975; 1 large cell cancer, H460; 1 squamous cell cancer, SK-MES-1) was detected by RT-PCR (A) and Western blot (B) and then analyzed with ImageJ (C). (D) The Wnt2 protein in the supernatant of the 5 aforementioned cell lines was detected by ELISA assay.

(*P* = 0.663). ROC curve analysis were performed to determine the cutoff value of serum Wnt2 level, which was set to 4.39 ng/mL to provide the maximum diagnostic accuracy (sensitivity, 40.52%; specificity, 87.69%).

The serum samples of 67 patients whose tumor tissues were stained in the aforementioned immunohistochemistry assays were collected and divided into two group according the cutoff value of serum Wnt2 levels, that is, ≤ 4.39 ng/mL group and > 4.39 ng/mL group. The distribution of Wnt2 IHC level of these patients according to serum Wnt2 level was shown in **Figure 3B**. A Spearman rank correlation test was run to evaluate the relationship between

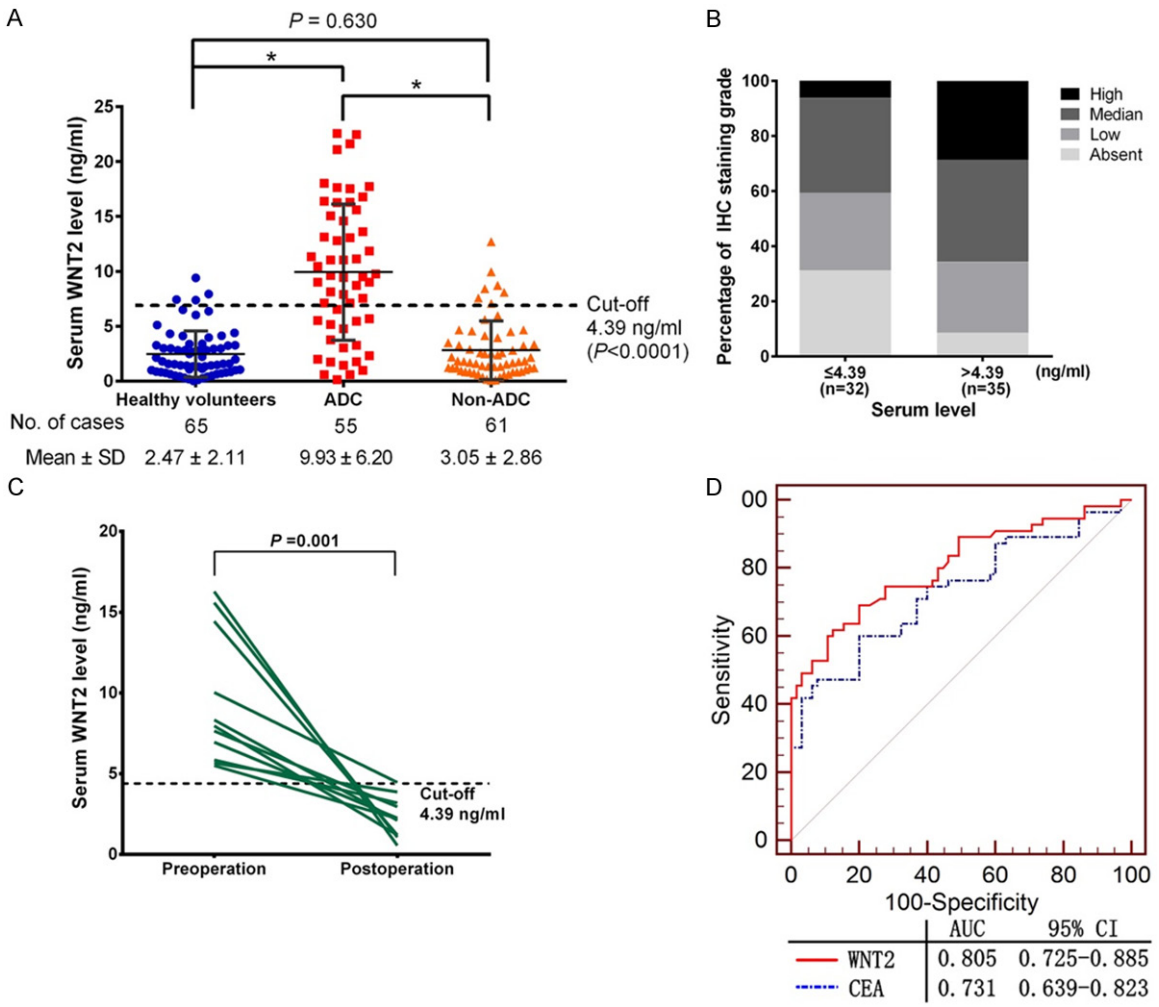


Figure 3. Serologic level of Wnt2 determined by ELISA experiments in patients with NSCLC and healthy volunteers. A: Serum Wnt2 levels of patients with NSCLC (patients were divided into ADC and non-ADC groups) and healthy controls. Black line indicates the cutoff value (4.39 ng/ml) which was determined by ROC curve in MedCalc programme. B: Distribution of Wnt2 IHC staining results of 67 NSCLC patients according to serum Wnt2 level. C: Serum levels of Wnt2 before and after radical resection of primary tumors in 12 NSCLC patients (P = 0.001). Black lines indicate cutoff values. D: ROC curve analysis of serum Wnt2 (Red line) and CEA (green line) as serum markers for adenocarcinoma of lung cancer. x-axis: 1-Specificity; y-axis, sensitivity. *: P < 0.05.

Table 3. The correlation between immunohistochemical results of Wnt2 and corresponding serum Wnt2 level in 67 patients with NSCLC

		IHC staining grade						
		Absent	Low	Median	High	Total	rs	*P*
serum level	≤ 4.39	10	9	11	2	32	0.344	0.004
	> 4.39	3	9	13	10	35		
	Total	13	18	24	12	67		

P values were calculated by the Spearman rank correlation test.

the serum Wnt2 level and the Wnt2 IHC level (**Table 3**). The results revealed that the serum level was correlated with IHC level in patients with NSCLC. We also compared the serum Wnt2 levels of 11 pairs of serum samples (before surgery and 1 month after surgery) from patients with NSCLC. Significant differences were noticed and the mean values were 9.48 ± 4.07 ng/mL and 2.28 ± 1.24 ng/mL respectively (**Figure 3C**). Altogether, these results suggested that the serum Wnt2 protein might derived from tumor cells.

To further evaluate the clinical significance of serum Wnt2 protein, we compared it with CEA, a conventional tumor marker for ADC, in the same set of serum samples from the 55

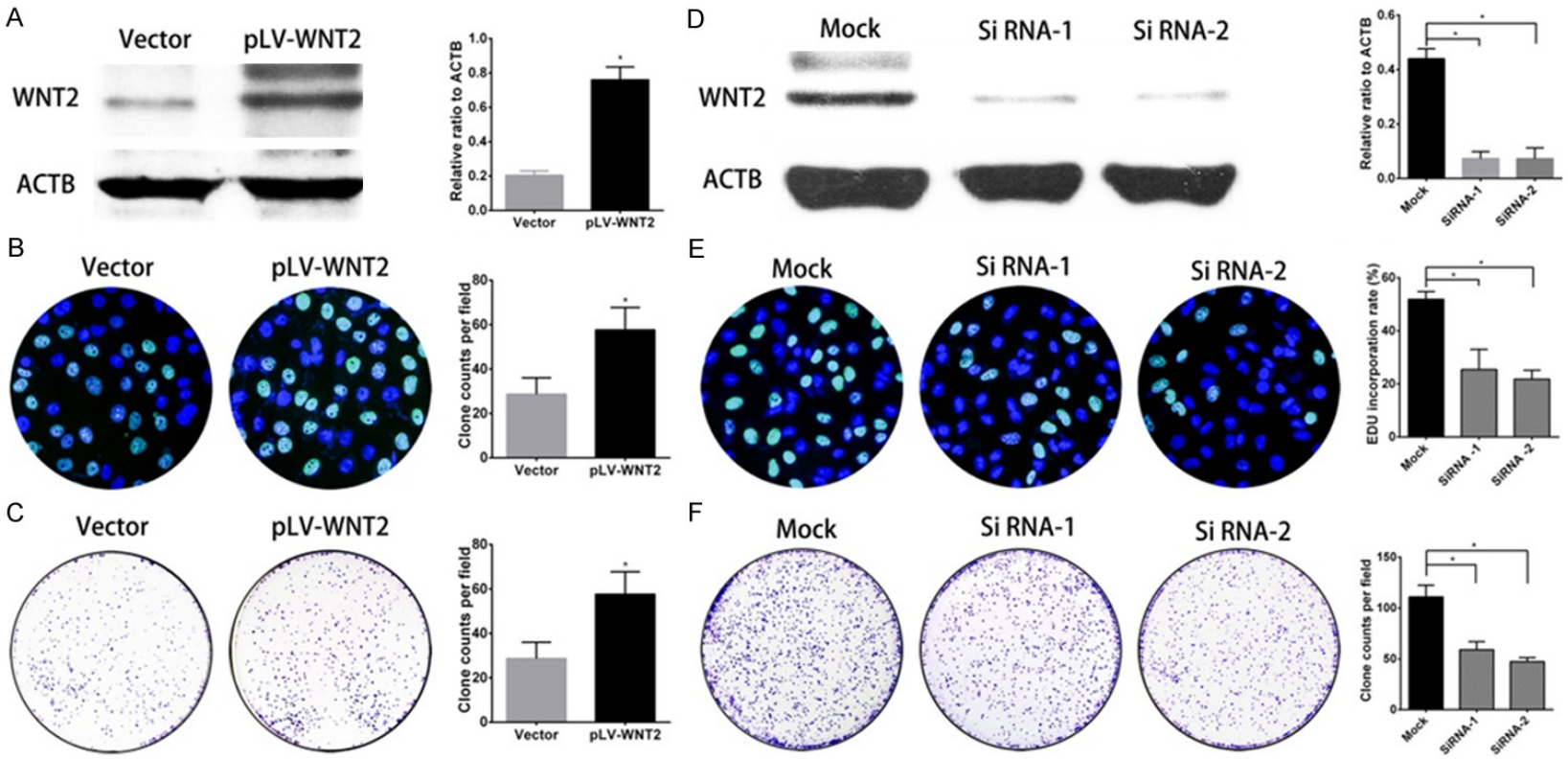
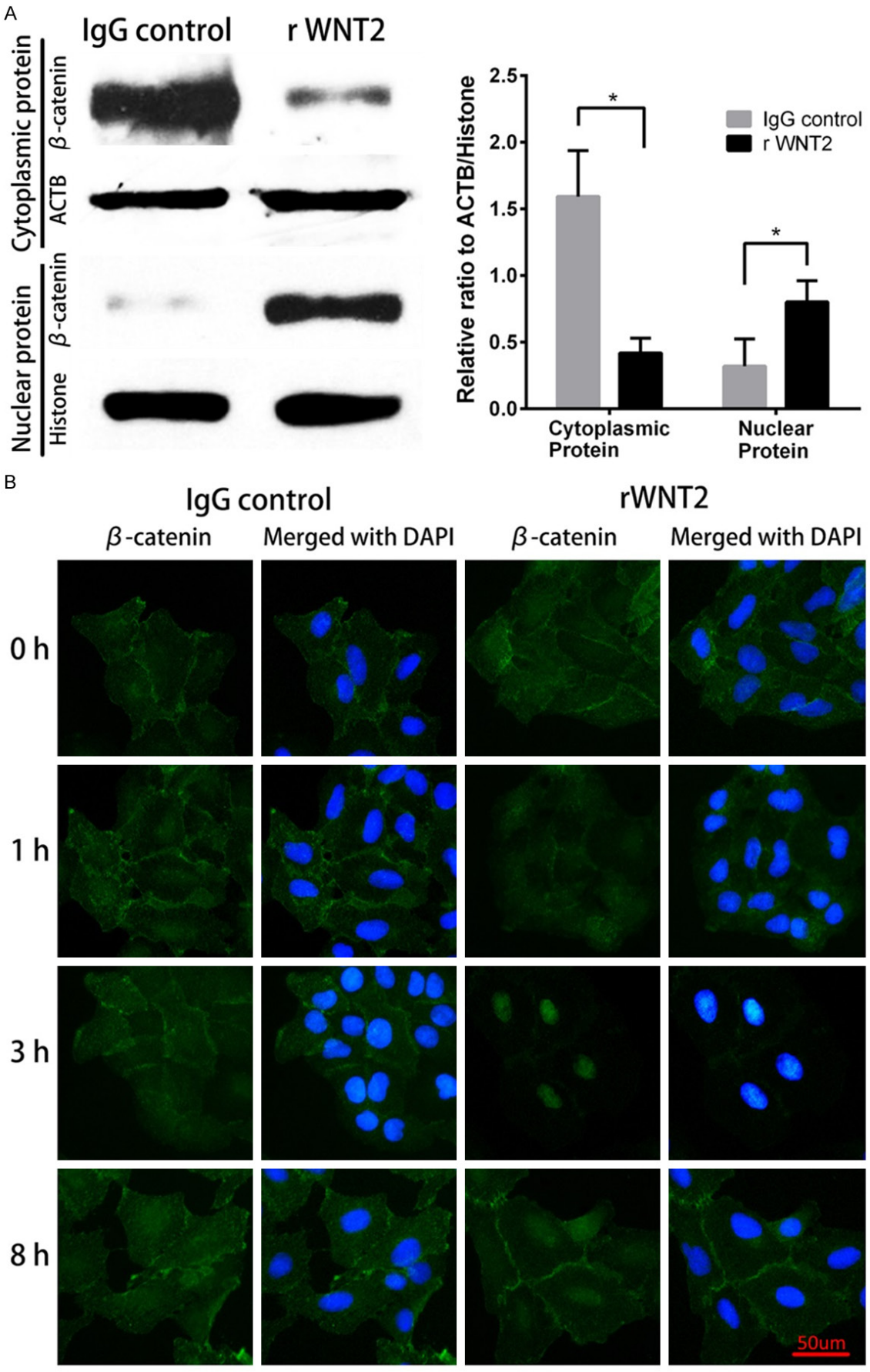


Figure 4. Enhanced or inhibited growth of lung cancer cells by stably overexpressing exogenous Wnt2 or siRNAs against WNT2. (A) H460 cells were stably transfected with pLV-WNT2 vector or empty vector as control via lentivirus. Western blot assays were used to verify the transfection efficiency. The growth rate of the cells was evaluated by the EDU incorporation assays (B) and colony formation assays (C). (D) Gene knockdown effect on WNT2 expression in H1299 cells by siRNAs against WNT2 and scramble siRNAs. Forty-eight hours after transient transfection, the growth rate of the cells was evaluated by the EDU incorporation assays (E) and colony formation assays (F). *: $P < 0.05$.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IgG control
r WNT2
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ACTB
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Relative ratio to ACTB/Histone
2.5
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Merged with DAPI
β-catenin
Merged with DAPI
0 h
1 h
3 h
8 h
50um

Figure 5. rWnt2 induces nuclear translocation of β-catenin and activates Wnt/β-catenin signalling pathway in H460 cells. A: Wild type of H460 cells were incubated with rWnt2 or IgG control at a concentration of 200 ng/mL for 3 hours and then the cytoplasmic (top) and nuclear (bottom) protein was extracted and detected by Western blot assays. The images were analyzed by ImageJ (right). *: $P < 0.05$. B: Representative immunofluorescence images demonstrate the translocation process of β-catenin from the membrane to nucleus in H460 cells incubated with rWnt2 but not with IgG control at a concentration of 200 ng/mL at the indicated time points. Antibody against β-catenin was labelled with green color and nuclei were labelled with blue color (DAPI). Original magnification, 400 ×; scale bars, 50 um.

patients with ADC and the 65 healthy controls by ELISA (**Figure 3D**). ROC curves were drawn and the results showed that the area under curve (AUC) of Wnt2 was slightly larger than that of CEA (difference between areas, 0.0738; SE, 0.0596; 95% CI, -0.0429-0.191; *P* = 0.0452).

Wnt2 overexpression promoted cell proliferation in NSCLC cells in vitro

To analyze the effect of Wnt2 on cell proliferation, the Wnt2-expressing plasmid was constructed and then transfected into H460 cells via lentivirus to stably overexpress Wnt2 protein. A vector plasmid was stably transfected into H460 cells as a control. The overexpression of Wnt2 in H460 cells was confirmed at protein levels by western blot experiments (**Figure 4A**). EDU incorporation assays revealed that the established H460 cell line expressing exogenous Wnt2 (H460-Wnt2) exhibited significant high DNA synthesis activity compared with control cells (**Figure 4B**). Then colony formation assays (**Figure 4C**) confirmed that H460-Wnt2 cells formed much more colonies than control cells.

siRNA against Wnt2 Inhibited growth of lung cancer cells in vitro

To disclose the role of tumor-derived Wnt2 in lung cancer cells, we evaluated the knockdown effect of endogenous Wnt2 expression by siRNA in A549 cells. A scramble siRNA sequence was used as a control. The siRNA effectively reduced the expression of Wnt2 (**Figure 4D**) and resulted in significant inhibition of DNA incorporation rate (**Figure 4E**) and reduced colony numbers (**Figure 4F**).

Wnt2 promotes cell proliferation via activating Wnt signaling pathway

To explore the potential molecular mechanism of the growth enhancing effect of Wnt2 on NSCLC, the Wnt/β-catenin signaling pathway in H460 cells was characterized. We incubated wild type H460 cells with rWnt2 or IgG control for 3 hours and then extracted the nuclear and cytoplasmic protein. Western blotting assays revealed that with rWnt2 treatment β-catenin level decreased in cytoplasm and increased in nucleus (**Figure 5A**). Subsequently immunofluorescence was done to visualize the localization of β-catenin at successive points in time (0, 1, 3 and 8 hour after the initiation of rWnt2). Prior to incubation with rWnt2, β-catenin was primarily located on the membrane of H460 cells (**Figure 5B**). After incubation with rWnt2 for 1 hour β-catenin on the membrane began to enter the cytoplasm of H460 cells. At 3 hour, β-catenin shifted into the nucleus. Intriguingly, β-catenin disappeared in the nucleus and reappeared in the membrane of H460 cells after 8 hour incubation with rWnt2. Nevertheless the nuclear translocation of β-catenin could be hardly observed when H460 cells were incubated with IgG control.

Wnt2 stimulates tumor growth in vivo

Ki67 was used as a proliferation indicator to probe whether Wnt2 expression level was associated with tumor growth activity in vivo. Consecutive sections of patients with NSCLC were stained with Ki67 and Wnt2 antibody (**Figure 6**). The results in **Table 4** revealed that higher Wnt2 IHC staining scores correlated with higher Ki67 labelling index scores (rs, 0.316; *P* < 0.0001). To determine whether overexpression of Wnt2 in tumor cells affects growth activity in vivo, 5×10^6 pLV-Wnt2 or pLV-vector H460 cells were injected subcutaneously into each Balb/c nude mouse (**Figure 7A**). As shown in **Figure 7B**, our results indicated that the growth activity of pLV-Wnt2 H460 cells was significantly higher than pLV-vector H460 cells. The ex vivo pLV-Wnt2 H460 tumors were significantly bigger than pLV-vector H460 tumors (**Figure 7C**). Subsequently, immunofluorescence staining of Wnt2 and Ki-67 was conducted to evaluate the association between Wnt2 and Ki-67

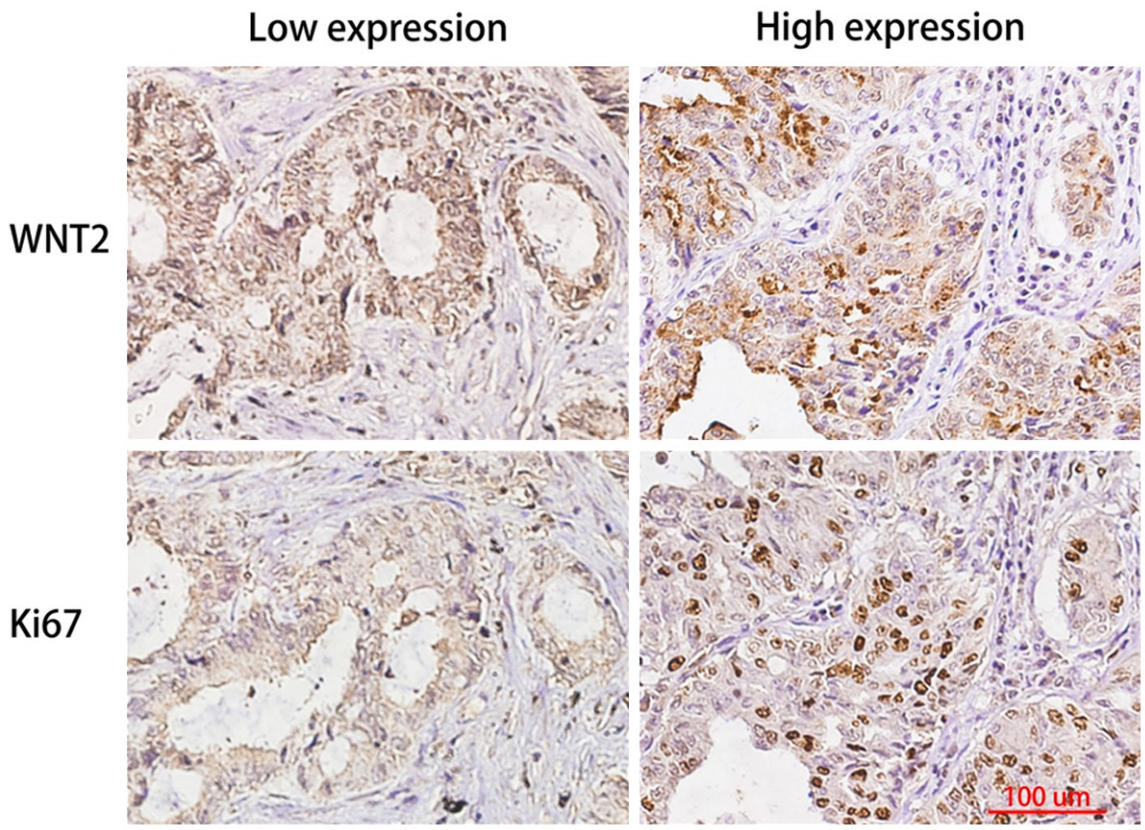

Figure 6. The correlation between Wnt2 and Ki67 expression in tumor tissues of NSCLC. Representative immunohistological images of Wnt2 and Ki67 correlative expression in consecutive sections from human NSCLC surgical specimens. Original magnification, 200 ×; scale bars, 100 mm.

Table 4. Statistical analysis of immunohistochemical results of Wnt2 and Ki67 labelling index in 264 human NSCLC surgical samples

		Ki67 index quartiles					rs	*P*
		First	Second	Third	Top			
		≤ 11.5%	11.6-19.8%	19.9-21.5%	> 21.5%	Total		
WNT2	-	20	59	10	9	98	0.316	< 0.0001
	+	8	26	28	13	75		
	++	11	15	12	21	59		
	+++	2	7	17	6	32		
	Total	41	107	67	49	264		

P values were calculated by the Spearman rank correlation test.

Figure 7D). As expected, pLV-Wnt2 H460 tumors showed significantly higher Ki-67 positive staining counts than the controls (**Figure 7E**).

Discussion

Wnt signaling pathway is a kind of highly conservative signal transduction pathway in the process of biological evolution. The Wnt proteins, consisting of a large family of secreted glycoproteins, play an essential role in normal development and organogenesis, including in embryonic development [19] and tissue repair [20]. The Wnt proteins have been identified to be key factors in the process of tumorigenesis [21]. As an important member of the WNT family, WNT2 has been reported as a protooncogene which has the ability to activate the WNT/β-catenin signalling pathway. The Wnt2 protein

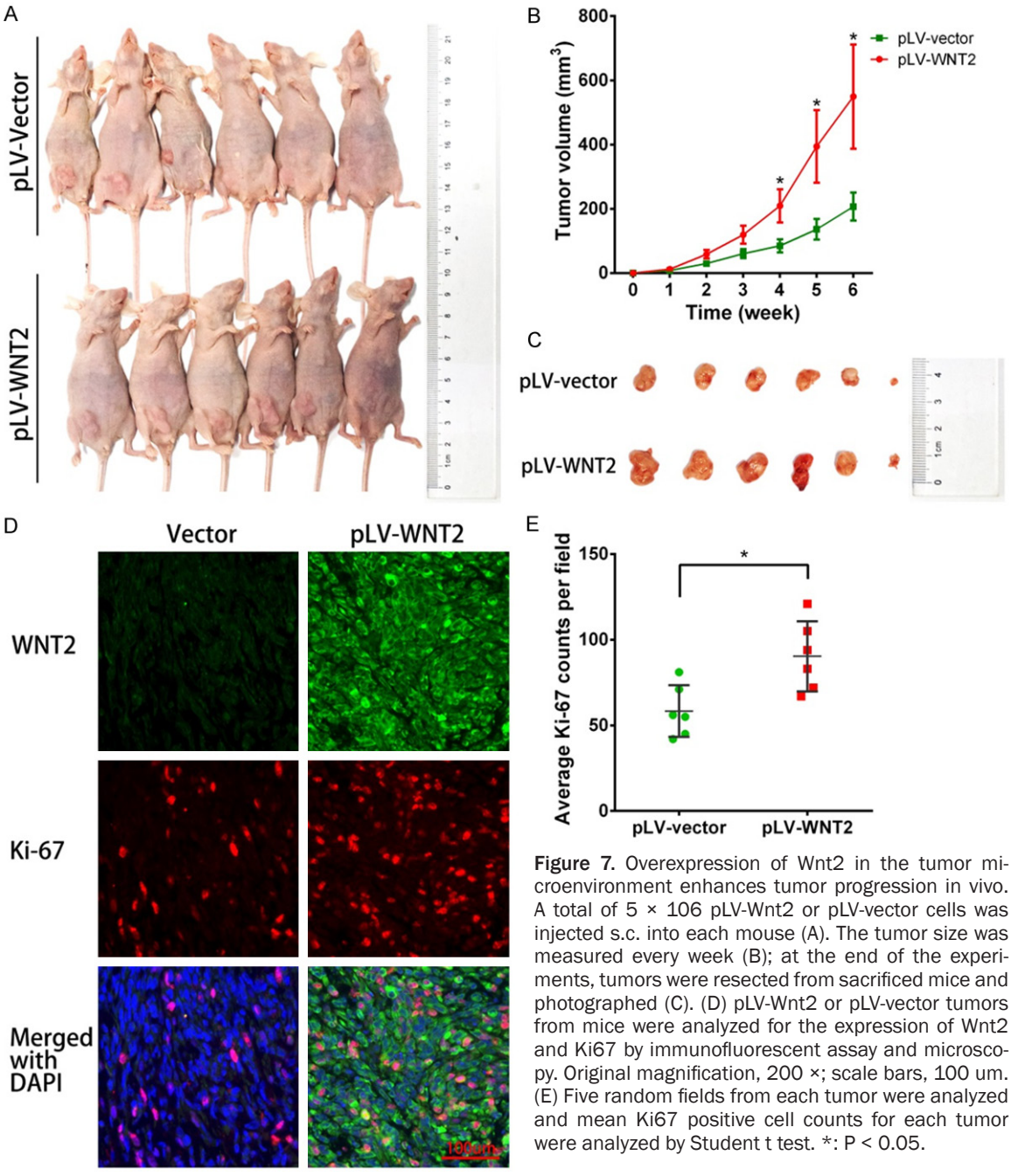

Figure 7. Overexpression of Wnt2 in the tumor microenvironment enhances tumor progression in vivo. A total of 5 × 106 pLV-Wnt2 or pLV-vector cells was injected s.c. into each mouse (A). The tumor size was measured every week (B); at the end of the experiments, tumors were resected from sacrificed mice and photographed (C). (D) pLV-Wnt2 or pLV-vector tumors from mice were analyzed for the expression of Wnt2 and Ki67 by immunofluorescent assay and microscopy. Original magnification, 200 ×; scale bars, 100 um. (E) Five random fields from each tumor were analyzed and mean Ki67 positive cell counts for each tumor were analyzed by Student t test. *: $P < 0.05$.

reported to be over-expressed in many types of tumors, such as gastrointestinal carcinoma [9], breast cancer [11], pleural mesothelioma [22] and pancreatic carcinoma [12]. Li Fu et al. [8] reported that Wnt2(+) cells could be detected in 42/51 (82.4%) of the primary oesophageal squamous cell carcinoma cases. Secreted Wnt2 could promote oesophageal cancer cell proliferation through activating the Wnt/β-catenin signalling pathway and then upregulating cyclin D1 and c-myc expression. They also found that Wnt2 could enhance cell migration and invasion ability by inducing epithelial-mesenchymal transition. Julien Mazieres et al. [22] performed Wnt-specific microarrays in normal pleura and malignant mesothelioma of the pleura (MPM) and found that among the 96 genes studied the most common event in MPM was the upregulation of Wnt2. They also demonstrated that inhibition of Wnt2 induced programmed cell death in MPM cells. In a study Liang You et al. [14] used RT-PCR and Western

blot experiments to demonstrate that Wnt-2 protein is overexpressed in freshly resected human NSCLC tissues. They found that blockade of Wnt2 by antibody or SiRNA could induce apoptosis in human NSCLC cell lines that overexpress Wnt-2 protein via downregulation of β-catenin and reduction in TCF-dependent transcriptional activity.

In the present study, we explored the clinical role of Wnt2 in NSCLC progression. We detected Wnt2 expression in 264 tumor sections and 181 serum samples. The IHC staining results revealed that Wnt2 was expressed in 62.9% of NSCLC tumor tissues and seldom in their corresponding normal lungs. Though Wnt2 expression could be detected in majority pathotypes of NSCLC, ADC tissues had significant more strong Wnt2 signal than non-ADC tissues ($P < 0.0001$). This distribution tendency is in line with that Wnt2 is usually reported to be positive in other adenocarcinomas. Additionally, our results showed that the Wnt2 expression level in NSCLC cancer tissues was significantly associated with pathologic T staging ($P = 0.001$). KaplanMeier analysis indicated that high Wnt2 level was significantly associated with poor prognosis. Wnt2 expression level was found to be a independent prognostic factor for both overall survival ($P = 0.049$) and relapse-free survival (0.002). These data suggest that Wnt2 might play an important role in the development and progression of NSCLC and could be used as a prognostic factor.

To evaluate whether Wnt2 was overexpressed in serum of patients with NSCLC, we did ELISA assays to detect serum Wnt2 levels and showed that the serum Wnt2 was significantly higher in patients with ADC than in healthy volunteers. However, there were no significant difference in serum Wnt2 level between patients with non-ADC and healthy controls. In addition, we verified the results in another Elisa assay. The serum Wnt2 level showed good correlation with its expression levels in primary tumor tissue in the studied patients. The concentration of serum Wnt2 was significantly decreased after surgical resection of primary tumors. What is more, AUC for serum Wnt2 levels was significantly larger than that for CEA (0.805 vs. 0.731), indicating that Wnt2 might be a better diagnostic marker for lung adenocarcinoma. Though further validation analysis was needed, the data presented here revealed a potential usefulness of Wnt2 as a serologic biomarker for lung cancer. To the best of our knowledge, this is the first study to report that Wnt2 expression has a strong prognostic value and diagnostic value for human NSCLC.

To validate our hypothesis that Wnt2 plays an important role in the progression of NSCLC, we upregulated and downregulated Wnt2 expression in cell lines and then performed proliferation assays in vitro. Both EDU corporation assays and colony formation assays demonstrated that elevated Wnt2 expression level led to enhanced proliferation rate and a blockage of Wnt2 expression resulted in decreased proliferation rate. To verify the potential molecular mechanism for Wnt2 proliferation-promoting effect on NSCLC tumor cells, we performed western blot assays and immunofluorescence assays to track β-catenin translocation in cells after Wnt2 stimulating. Indeed, our results revealed that β-catenin was shifted into the nucleus from the cytoplasm. Subsequently the proliferation-promoting effect of Wnt2 was verified in vivo experiments. In primary tumor tissue the expression level of Wnt2 had good correlation with that of Ki67, which was usually used as an indicator for proliferation [23, 24]. In animal experiments, tumor cells overexpressing Wnt2 showed faster growth rate in vivo than the control cells.

In summary, we report that Wnt2 might enhance the proliferation of NSCLC tumor cells by activating WNT/β-catenin signaling pathway and could be used as both a potential serological diagnostic biomarker and a tissue prognostic biomarker for NSCLC.

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Table S1. Clinicopathological parameters of 264 patients with NSCLC

Clinicopathologic variable	No. (%)
Gender	
Male	163 (61.7)
Female	101 (38.3)
Age, y	
< 65	171 (64.8)
≥ 65	93 (35.2)
Pathologic type	
ADC	134 (50.8)
Non-ADC	130 (49.2)
pT stage	
T1+T2	229 (86.7)
T3+T4	35 (13.3)
pN stage	
N0	28 (10.6)
N1+N2	236 (89.4)
Differentiation	
Well+Moderate	177 (67.0)
Poor	87 (33.0)