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Regulation of NSCLC Cell Proliferation By MARCH7 Via the NF-κB and Wnt/β-Catenin Signaling Pathways

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Abstract

The aim of the study was to explore the role of the E3 ubiquitin ligase MARCH7 in the development of non-small-cell lung cancer (NSCLC) and to explore the underlying molecular mechanism.

Western blot and immunohistochemistry results showed that the expression of MARCH7 in NSCLC cancer tissues was higher than that in paracancerous tissues. Tissue microarray staining results and clinicopathological parameters of NSCLC patients revealed that MARCH7 expression was closely related to TNM stage, degree of tumor differentiation and lymph node metastasis of NSCLC patients. Furthermore, univariate and multivariate analyses and survival curve analysis showed that high expression of MARCH7 was associated with poor prognosis.

In vitro, siRNA was constructed and transfected into A549 cells to inhibit the expression of MARCH7. The CCK-8 assay indicated that the growth rate of tumor cells in the interference group was reduced. The number of colonies and cells in the interference group decreased in the plate clone formation experiment. Flow cytometry showed that G0/G1 phase cells were predominantly increased after blocking endogenous MARCH7 expression, and G0/G1 phase arrest occurred in A549 cells. The reporter gene activity of the NF-κB signaling pathway and Wnt/β-catenin signaling pathway was reduced, as validated by a double luciferase reporter gene assay. Western blot analysis showed that the expression of NF-κB P50, NF-κB P65 and β-catenin was decreased, while the expression of E-cadherin was elevated.

In vivo, MARCH7-overexpressing virus was constructed and transfected into A549 cells and then subcutaneously injected into nude mice. It was demonstrated that the tumor volume was significantly larger in the MARCH7 overexpression group than in the control nude mice during the same period. Elevated expression of PCNA and Ki-67 was observed in the tumor mass of the MARCH7 overexpression group, as measured by immunohistochemical analysis, accompanied by enhanced levels of NF-κB P50, NF-κB P65 and β-catenin, as detected by Western blot. These results provide a new idea for the experimental basis for the treatment of NSCLC in the future.

Introduction

Lung cancer currently has the highest incidence and mortality of malignant tumors worldwide. [1, 2] Based on histopathological phenotypes, lung cancer can be divided into two types, namely, small cell lung cancer and non-small cell lung cancer (NSCLC), and the incidence rate of the latter accounts for 85% of the total number of lung cancer cases, with 20%-30% being squamous cell carcinoma (SCC) and 40%-50% being adenocarcinoma (ADC)[3]. In recent years, the treatment of NSCLC has been continuously progressing, as has its efficacy, because of the strengthening of tobacco control, the improvement of surgical procedures and molecular targeted drugs, and the advent of various immunotherapy drugs[4, 5]. Despite the advances mentioned above, a remarkable proportion of NSCLC patients still develop recurrence or metastasis or have a low therapeutic response to conventional chemotherapy[6, 7]. Hence, it is urgently necessary to seek novel therapeutic targets of NSCLC, which could potentially have important

clinical implications. Currently, numerous studies have found that multiple signaling pathways, such as MAPK, PI3K/AKT and Hedgehog, are aberrantly involved in the growth, invasion, and metastasis of NSCLC, among which the NF-κB pathway and Wnt/β-catenin pathway are found to play a critical role in NSCLC progression[8].

Meanwhile, membrane-associated ring finger (C3HC4) 7 is recognized as a potential negative regulator of the NF-κB pathway and Wnt/β-catenin pathway[9]. The superfamily of membrane-associated ring fingers (C3HC4) consists of 11 members that participate in the regulation of multiple immune responses, protein sorting and maturation of spermatozoa. [10, 11]. MARCH7 is a member of the family mentioned above, and its molecular structure is composed of 690 amino acids and contains zinc finger domains. According to the literature reports, MARCH7 is mainly involved in the regulation of the NF-κB pathway and Wnt/β-catenin pathway in two ways. First, by facilitating the nuclear import of p50 and p65, the activity of the NF-κB signaling pathway is thus regulated by MARCH7. Second, MARCH7 upregulates β-catenin expression and facilitates its nuclear import to regulate c-myc, sp5 and lef1, which are target genes of the Wnt/β-catenin signaling pathway. Therefore, it is hypothesized that MARCH7 may participate in the proliferation of NSCLC and determine malignant progression by regulating the NF-κB and Wnt/β-catenin signaling pathways.

On the basis of the abovementioned literature reports, our research group employed TCGA (The Cancer Genome Atlas) for data analysis, and we found that MARCH7 was highly expressed in adenocarcinoma and lung squamous cancer, especially in the latter. We also confirmed that MARCH7 was upregulated in NSCLC tissues compared with adjacent normal tissues. The aforementioned observations found that MARCH7 probably plays an important role in the development of NSCLC.

This study intends to collect clinical samples of NSCLC patients and examine the association between MARCH7 expression and clinicopathological parameters of NSCLC. In addition, the cell proliferation of NSCLC influenced by MARCH7 will be assessed at the cellular level *in vitro*, aiming at exploring its molecular mechanisms. Finally, MARCH7 overexpression will be assessed in nude mice to analyse its effects on tumor growth. This topic will explore the role and molecular mechanism of MARCH7 in NSCLC and serve as an experimental basis and theoretical foundation for the novel prognostic assessment of NSCLC and molecular markers in patient follow-up and emerging targets to treat cancer.

Materials And Methods

sample collection

The histological types were kept by the Department of Pathology, Hai 'an City People's Hospital between January 2013 and January 2015. These tissue specimens were obtained from patients with primary NSCLC whose survival time was no less than 3 months, and they had not received preoperative chemotherapy, radiotherapy, targeted therapy or immunotherapy before surgical resection. Then, 6 NSCLC tissue specimens were randomly chosen and fresh-frozen in liquid nitrogen; adjacent normal tissues located 3 cm from the carcinomas were collected for use as a control group. The survival time was

calculated from the date of diagnosis until death or the last follow-up, which was January 1, 2020. This study was approved by the Ethics Committee of Hai 'an City People's Hospital. All patients provided informed consent and signed the consent form. The staging of these patients with NSCLC was based on the 8th Edition of TNM classification of the Union for International Cancer control (UICC) and American Joint Committee on Cancer (AJCC). Specific clinical data of these patients are described below (Table 1).

Table 1

Clinicopathological features		Number Cases
Ages	<60	112
	≥60	88
Gender	Male	123
	Female	77
Pathological Type	SCC	75
	ADC	100
	Other	25
Degree of Differentiation	high-moderate differentiation	109
	low differentiation	91
Lymph Node Metastasis	yes	85
	no	115
TNM Stage	stage Ⅰ+Ⅱ	135
	stage Ⅲ+Ⅳ	65

Western Blot Detection, Tissue Chip Preparation and Immunohistochemical Staining

Proteins were extracted from NSCLC cancer tissue, paracancerous tissue and cell lines that were preserved in liquid nitrogen and subjected to SDS–PAGE. After that, the proteins were transferred to a PVDF membrane, which was incubated with MAb MARCH7 antibody (diluted at 1:1000). After the addition of the developing solution, the PVDF transfer membrane was exposed to X-ray film in a dark room. Then, the slides were left to dry and scanned for analysis with a gel imaging system to determine the target protein bands. The gray value ratio of the target protein to that of the internal reference protein was regarded as the relative expression of the target protein.

The collected NSCLC tissue samples were chosen for tissue chips, and each sample was taken at 2 sites. NSCLC tissue sections were immunohistochemically stained with MARCH7 antibody (Abcam), and

positive staining was indicated by buffy granules present in the cell membrane or cytoplasm. Gene expression of MARCH7 was evaluated according to the semiquantitative integration method. Then, we screened specimens for which positive staining was scored relatively high and randomly chose five 400-fold fields of view. From each view, 100 tumor cells were counted. Positive staining was scored according to Tables 2 and 3 below, and the total score of each specimen was calculated using the formula (Scores=2×3). A score ≤ 3.5 was identified as the low expression group, and a score >3.5 was identified as the high expression group.

Table 2The degree of staining

shades of staining	Scores
Negative staining	0
Pale-yellow	1
Yellow	2
Tan or Brown	3

Table 3number of the positively stained cells

The ratio of positively stained cells%%	Scores
≤5%	0
6%-25%	1
26%-50%	2
51%-75%	3
75%	4

Plasmid Construction, Cell Transfection and Cell Experiments

Information on MARCH7 gene transcripts and CDS was collected from the NCBI database (https://www.ncbi.nlm.nih.gov/gene). Three siRNA sequences of MARCH7 were designed and synthesized according to the full-length sequence of MARCH7, and nonspecific siRNA was synthesized at the same time (sequences are provided in the supplemental material). Plasmid-liposome complexes and the A549 cell line were coincubated with A549 stably transfected cells serving as the experimental group and untransfected cells serving as blank controls. To investigate the effect on cell proliferation and the cell cycle, CCK-8 assays, flat plate colony formation tests and flow cytometric analysis were performed. Finally, we used luciferase reporter gene assays to examine the downstream signaling pathway activation of NF-κB and Wnt/β-catenin and then measured the molecular signaling pathway expression with Western blot analysis.

Animal models

Thirty specific pathogen-free female BALB/c mice (6–8 weeks old, 18-20 g) were purchased from SlakeLaboratory Animal Company, Shanghai, China. All the experimental mice were raised at the SPF level of the Animal Experiment Center of Nantong University. Animal protocols were approved by the ethics committee of this university. All animal sacrifices, treatments and postoperative animal procedures were performed in strict accordance with the regulations of experimental animal administration and the animal ethical committee of Medical College.

According to the pre-experiment, A549 cells were infected with the desired virus at an MOI of 50 (specific details are provided in the supplemental materials). The A549 cells were made into a cell suspension whose density was adjusted to 1 × 107 cells/mL and wasinoculated subcutaneously into the armpit of the nude mouse. The skin entrance point was 1 cm away from the injection point, forming a protruding cutaneous mound to prevent the suspension from leaking out. Before and after injection, 75% alcohol was used to disinfect the injection site. Nude mice were randomly allocated into 3 groups: tumorigenic, tumorigenic+unloaded virus, and tumorigenic+MARCH7-overexpression groups (three mice per group). After injection, the spirit and dietanddefecation habits of nude mice were observed daily. Thelongandshortdiameterofthetumor was measured and recorded every 2 days, then a tumorgrowth curve was plotted, with time asthe abscissa andtumorvolumeasthe ordinate (tumorvolumewas calculated using the formula: Tumor volume (V)=1/[(2*longdiameter*short diameter)^2]). The nude mice were sacrificed after 21 days, and all the tumours were isolated. Then, the expression of PCNA and Ki-67 was detected by immunohistochemistry to assess tumor proliferation. Finally, Western blot analysis was performed to examine the changes in Wnt/β-catenin signaling pathway-related gene expression.

Statistical analysis

All the experiments in this study were repeated three times independently. The counting data were expressed as (%). Differential analysis was performed using the chi-square test or Fisher's exact test; metric data were expressed as the mean ± standard deviation ($\bar{x} \pm S$), and paired Student's t test (for two groups) or variance test (three or more groups) was performed on differential analysis. Inspection standards taking $\alpha = 0.05$. A p value < 0.05 was considered statistically significant. Correlations were analysed using the Spearman correlation test. Survival curves were plotted by using the Kaplan–Meier method. The survival difference of NSCLC patients was evaluated using the log-rank test. All the data were used for statistical analysis with SPSS 20.0 software.

Results

Overexpression of MARCH7 in NSCLC Cancer Tissue Detected by Western Blot and Immunohistochemical Analysis

Western blotting was conducted to detect MARCH7 expression in three pairs of NSCLC cancer tissues and adjacent tissues, which showed that the expression of MARCH7 in NSCLC was significantly higher than

that in paracancerous tissues. Statistical analysis suggested that the relative gray values of the MARCH7 protein bands of NSCLC cancer tissue were also significantly higher than those of paracancerous tissues (*p<0.05).

Carcinoma tissues containing both cancer and adjacent tissues were obtained from patients with NSCLC, which was stained by H&E with histopathologically proven NSCLC and made into paraffin blocks (see Fig. 1B). Immunohistochemical staining was applied, and the results showed that MARCH7 was predominantly expressed in immune cells, and the levels of MARCH7 expression were higher in NSCLC cancer tissues than in paracancerous tissues (*p<0.05).

Further Evidence from a Tissue Microarray on High Expression of MARCH7 in NSCLC

The tumor tissues collected from 200 cases of NSCLC were stained by H&E and made into an NSCLC tissue microarray. Twenty-five NSCLC cases were removed because of tissue depletion of sections, incomplete records of clinicopathological data and loss to follow-up. MARCH7 expression in cancerous and adjacent tissues of 175 NSCLC cases was assessed by immunohistochemistry. By statistical analysis, the MARCH7 high-expression group comprised 38.29% of the patients, and the low expression group included 11.43% of the patients. Both differences showed statistical significance with p<0.001.

Association between MARCH7 Expression and Clinical Pathological Parameters in 175 Patients with NSCLC

According to our findings, the expression of MARCH7 varied significantly among the NSCL tissues at stages I-II and at stages III-IV; the higher the stage of the cancer was, the higher the expression of MARCH7 (p<0.05). The expression of MARCH7 was associated with tumor differentiation; the lower the differentiation was, the higher the MARCH7 expression was (p<0.05). In addition, the expression of MARCH7 was associated with lymph node metastasis; the expression of MARCH7 was higher in patients with lymph node metastasis than in those without lymph node metastasis (p<0.05). However, its expression showed no association with sex, age, histological type, smoking habit or tumor size. (See Table 4)

Table 4 *Association between **MARCH7 Expression** and Clinical Pathological Parameters **of 175** Patients with NSCLC*

Variate		Cases	MARCH7 Expression		*P*
			Low Expression	High Expression	
Age	<60	100	68	32	0.118
	≥60	75	40	35	
Gender	Male	107	67	40	0.216
	Female	68	41	27	
Smoking	Yes	106	66	40	0.374
	No	69	42	27	
TNM	□-□	120	98	22	<0.001*
Stage	□-□	55	10	45	
Tumor Size	<3 cm	104	61	43	0.105
	≥3 cm	71	47	24	
Histological Type	SCC	68	40	28	0.083
	ADC	87	57	30	
	Other Types	20	11	9	
Degree of Tumor differentiation	Low	79	20	59	<0.001*
	High	106	88	8	
Lymph Node Metastasis	Yes	77	26	51	<0.001*
	No	98	82	16	

Note: “Other Types” contains lung adenosquamous cell carcinoma, large cell carcinoma, lung mucoepidermoid carcinoma and carcinosarcoma. The P value is the result of the statistical analyses of the comparison between squamous cell carcinoma and adenocarcinoma. *indicates statistically significant difference p<0.05.

Statistical Analysis of Risk factors for Prognosis of Patients with NSCLC

Univariate analysis of the level of MARCH7 expression
and clinicopathological parameters was performed with SPSS 20.0. As is shown in Table 5. The overall survival of NSCLC patients showed no association with patient sex, age, histological type, or smoking habit but was associated with MARCH7 expression, tumor size, tumor differentiation, lymph node metastasis and tumor-node-metastasis (TNM) stage (P<0.05). Factors with univariate analysis P value <

0.05 were used for further multivariate analysis, of which the result shows that NSCLC patients with high-expression MARCH7 demonstrate poor prognostic performance (See Table 6).

Table 5Univariate Analyses of the Clinicopathological Parameters of NSCLC Patients

Variate	Univariate Analyses		
	HR	95%CI	*P*
Age<60vs≥60	1.303	0.956-1.852	0.241
Gender (Male or Female)	1.573	1.311-1.969	0.154
Smoke or Not	0.882	0.442-1.428	0.175
TNM Stage-vs-	2.182	1.721-2.849	0.003*
Tumor Size<3 cmvs≥3 cm	1.431	1.021-2.153	0.025*
Degree of Tumor DifferentiationHigh vs Low	0.699	0.437-1.052	<0.001*
Lymph Node MetastasisYes vs No	0.683	0.448-1.152	<0.001*
MARCH7 ExpressionLow vs High	1.762	1.338-2.415	0.002*

Table 6Multivariate Analyses of the Clinicopathological Parameters of NSCLC Patients

Variate	Multivariate Analysis		
	HR	95%CI	*P*
TNM Stage-vs-	1.743	1.149-2.632	0.165
Tumor Size <3 cmvs≥3 cm	1.157	0.814-1.548	0.016*
Degree of Tumor DifferentiationHigh vs Low	0.682	0.473-1.228	0.218
Lymph Node MetastasisYes vs No	0.632	0.473-1.264	<0.001*
MARCH7 ExpressionLow vs High	1.683	1.259-2.017	0.013*

Note: If univariate analysis is statistically significant at P<0.05, multivariate analysis will be performed to investigate its independent prognostic value. If multivariate analysis was still statistically significant at P<0.05, the variate was proven to be an independent prognostic predictor. (CI: 95% confidence interval; HR: hazard ratio; *indicates statistically significant difference p<0.05)

Analysis of Kaplan–Meier Survival Curves

Immunohistochemical staining was performed using paraffin sections of carcinoma tissue from 175 NSCLC patients, which were grouped according to the level of MARCH7 expression. The survival rate of the high expression group was significantly lower than that of the low expression group, as shown in Figure 3 (P<0.05).

Association of MARCH7 Expression and Proliferation among NSCLC Cell Lines

Western blot analysis was used to detect the level of MARCH7 expression in NSCLC cell lines (see Fig. 4A), and MARCH7 expression was found in A549, H1975, SPC-A-1 and XLA-07 NSCLC cell lines and was significantly higher than that in normal lung bronchial epithelial BEAS-2B cells ($P<0.05$). Therefore, MARCH7 was broadly expressed throughout the NSCLC cell line and was capable of being expressed at higher levels in the NSCLC cell line than in the bronchial epithelial cell line. According to indices such as the ratios of viral infection and proliferation rate, the A549 cell line was adopted as the research object in the following study.

In subsequent *in vitro* experiments, the A549 cell line was cultured for 72 h in starvation medium. Then, serum was added at different time intervals (6 h, 12 h, 24 h, 48 h), and cellular proteins were harvested at the time points mentioned above. The Western blot analysis results showed that MARCH7 expression gradually increased with prolonged cultivation time, as well as the expression of the proliferation markers PCNA and CyclinA. The expression trends were similar among the three, with temporal correlations represented. All data above indicated that MARCH7 was intimately associated with tumor cell proliferation.

The Effects of MARCH7 on the Proliferation of NSCLC Cells

To validate the association of MARCH7 with tumor cell proliferation, the A549 cell line was selected as an experimental model. The level of MARCH7 expression in A549 cells was measured by qRT–PCR (see Fig. 5A). Compared with control siRNA transfection, the expression level of MARCH7 visibly decreased after MARCH7-siRNA transfection, and optimal effects of an intervention were represented in the MARCH 7-siRNA-1709 group. Thus, the MARCH7-siRNA-1709 sequence was adopted in the subsequent functional experiments.

After the protein expression of MARCH7 was successfully interfered with, the effects of the change in MARCH7 expression level on the proliferation of the NSCLC cell line were detected by a CCK-8 kit. The results showed that the tumor growth rate of the MARCH7 siRNA group was significantly slowed (see Fig. 5B), which suggested a significant inhibition of A549 cell proliferation among NSCLC cell lines by blocking MARCH7.

MARCH 7-siRNA-1709 was transfected into cells, which were then Giemsa stained. The number of cell colonies was recorded ($\geq$50 cells), and data are presented as colony formation rates. After the inhibition of MARCH7 expression in A549 cells, the cell colony level decreased, as did the cell level, which suggested that there appeared to be an association between MARCH7 and A548 cell colony formation (see Fig. 5C).

Flow Analysis of Cell Cycle:

After transfecting MARCH 7-siRNA-1709, the cell cycle was measured with PI staining. The MARCH7-siRNA group had a higher proportion of G0/G1 cells than the control group ($P<0.05$), which indicated that

the blockade of endogenous MARCH7 expression led to an increase in cells in G0/G1 and G0/G1 phase arrest of A549 cells (see Fig. 5D).

Possible Mechanisms by which MARCH7 Regulates Cell Proliferation in NSCLC

To validate that MARCH7 possibly regulates cancerous cell proliferation by regulating the Wnt/β-catenin and NF-κB pathways, A549 cell lines were transfected with MARCH7-siRNA, and the activity of Wnt/β-catenin and NF-κB signaling pathway reporter genes was detected by dual-luciferase reporter gene assay. The results showed that the activity of NF-κB and TopFlash Reporter was significantly suppressed compared with that of the control group ($P<0.001$, see Fig. 6A). The expression levels of the two signaling molecules of the signaling pathway were then examined with Western blot, and the results showed that the expression levels of NF-κB P50, β-catenin and NF-κB P65 decreased, while E-cadherin showed the opposite trend (see Fig. 6B).

The PCNA and MARCH7 expression levels in A549 cell lines visibly decreased after potent inhibitor of the NF-κB pathway (PDTC) was added, and the trend continued as the concentration of PDTC was increased (Fig. 6C). Inhibition of P50 or P65 led to a decrease in MARCH7 and PCNA expression levels, while inhibition of both P50 and P65 led to a further decrease in MARCH7 and PCNA expression levels ($P<0.05$, see Fig. 6D). The results mentioned above suggested that MARCH7 was capable of regulating cancerous cell proliferation by regulating the Wnt/β-catenin and NF-κB signaling pathways.

The Effect of MARCH7 Overexpression on Tumor*in vivo*

The results of the study showed that all nude mice developed tumor nodules at the injection site (see Fig. 7A). Compared with the control group in the same period, tumor volume was significantly increased in the MARCH7 overexpression group, in which tumor growth began accelerating at day 10. The results mentioned above illustrate that MARCH7 promotes tumor growth *in vivo*.

Table 7: The Size of Tumors from Subcutaneous Tumor Formation Assay in Nude Mice

Group	n	8d	12d	15d	19d	21d
Control Group	3	17.29±0.4	55.79±8.2	73.25±7.8	139.1±14.6	158.9±8.75
EmptyVector Group	3	16.11±3.4	49.89±10.1	73.67±2.3	136.9±20.5	159.9±17.2
Virus Group	3	18.85±2.1	78.96±9.9*	103.1±12.3**	159.5±17.9**	196.9±8.82**

Note: *indicates a statistically significant difference $p<0.05$

Immunohistochemical analysis was performed in xenograft tumors, and the results showed that the expression levels of proliferation markers PCNA and Ki-67 in tumors of the MARCH7 overexpression group significantly increased (see Fig. 8), which suggested that MARCH7 in vivo promoted cancerous cell proliferation.

Western blot analysis of subcutaneous xenografts showed that the expression of NF-κB p50 and NF-κB p65 significantly increased in the MARCH7 overexpression group compared with the vector groups, and the expression of β-catenin also visibly increased compared with the control group (see Fig. 9).

Discussion

This study found that MARCH7 presented a high expression level in NSCLC tumor tissue, suggesting that MARCH7 may be a cancer-promoting gene. We also found that the more severe the lymphatic metastasis was, the later the stage of cancer and the higher the expression of MARCH7, which indicated that the expression level of MARCH7 may be closely associated with the growth and prognosis of NSCLC and that MARCH7 may be involved in regulating the development and progression of NSCLC. Thus, we speculated that MARCH7 would be a novel biomarker for monitoring disease conditions and estimating prognosis or a possible therapeutic target.

The survival curve demonstrated that the overall survival of NSCLC patients with high MARCH7 expression was significantly lower than that of those with low expression. These preliminary findings showed that high MARCH7 expression was related to the poor prognosis of NSCLC patients, which was concordant with the findings of previous studies on the effects of MARCH7 on cervical and ovarian cancer. Thus, we speculated that MARCH7 may be involved in the regulation of the malignant biological behavior of NSCLC.

To further verify our conjecture, the MARCH7 gene in A549 cells was silenced *in vivo*, and the cell proliferation and clone formation ability of A549 cells were observed to be significantly decreased, which proved that MARCH7 may play an important role in cancer promotion and maintenance of the malignant phenotype of tumors.

Activation of the NF-κB signaling pathway is involved in the tumorigenesis of several cancers, including lung, breast, cervical, gastric, and prostate cancers; in addition, NF-κB is also expressed in small cell lung cancer and non-small-cell lung cancer, inhibits apoptosis, promotes angiogenesis, proliferation and metastasis, and participates in tumorigenesis[12]. In epithelial ovarian cancers, MARCH7 is imported into the nucleus by regulating the activity of the NF-κB signaling pathway and the activation of the pathway pathway molecule, thus regulating the proliferation of ovarian cancer cells. Therefore, we speculated that MARCH7 regulates the proliferation of cancer cells by the NF-κB signaling pathway in NSCLC. To verify this conjecture, we performed a dual luciferase reporter gene assay, and MARCH7 with low expression was found to visibly inhibit the activity of the NF-κB signaling pathway luciferase gene. We also found that after the interference of MARCH7 expression, the protein levels of P65 and P50 decreased, which suggested that MARCH7 was capable of participating and mediating NF-κB signaling, thereby regulating the malignant biological behavior of NSCLC.

What is the specific biochemical mechanism by which MACRH7 activates NF-κB? E3 ubiquitin ligases can rapidly recognize the phosphorylation of IκB serine residues and result in the polyubiquitination modification of IκB, which in turn causes IκB to be degraded by the ubiquitin-dependent proteasome. NF-

κB is thus untethered by the degradation process above, exposes its nuclear localization signal (NLS), and is imported into the nucleus to activate the transcription of target genes[12]. However, there is still a need for further research to prove that MARCH7, an E3 ubiquitin ligase, activates NF-κB through the ubiquitin modification mentioned above. In the progression of malignancy, the NF-κB signaling pathway can be aberrantly activated at the inflammatory response phase and accelerate the malignant transformation of normal cells[12]. NF-κB, as a transcription factor, can be involved in mediating the transcriptional activity of various cancer-promoting genes and plays an important role in the genesis and evolution of cancer tumors. Therefore, we studied the effects of the NF-κB signaling pathway on the expression of MARCH7. The NF-κB inhibitor PDTC was added to A549 cells, and the results showed that MARCH7 expression decreased significantly and that the inhibitory rate of MARCH7 expression increased as the concentration of PDTC was increased. P50 and P65, subunits of NF-κB, were silenced by siRNA, and then the protein expression level of MARCH7 decreased, which suggested that NF-κB was capable of regulating the expression of the MARH7 gene.

In addition, Wnt signaling pathways include β-catenin-dependent canonical (Wnt pathway) and β-catenin-independent noncanonical (PCP pathway and Wnt/calcium pathway) pathways, and the former takes up the majority of pathways in cancer progression[13–14]. Once the transduction signaling pathway is overactivated, Wnt binds to frizzled transmembrane receptors, and glycogen synthase kinase-3β (GSK3β) is inhibited, leading to dephosphorylation and stabilization of β-catenin. Then, β-catenin accumulates in the nucleus, where it interacts with coregulators of transcription, including T cell factor/lymphocyte enhancer factor (Tcf/Lef), and functions as an activator of transcription[15–16]. Studies have proven that MARCH7 is a potential regulatory factor of the NF-κB and Wnt/β-catenin signaling pathways. Previous studies have shown that MARCH7 can affect NSCLC cell proliferation via the NF-κB signaling pathway. Therefore, as coregulatory factors, can MARCH7 regulate NSCLC cell proliferation via Wnt/β-catenin? Our research found that after interference of NSCLC expression, the activity of Wnt/β-catenin signaling pathway reporter gene significantly decreased, so did the expression of β-catenin; and E-cadherin expression increased which inhibits the activation and importation into nucleus of β-catenin. The results above indicated that MARCH7 was associated with the malignant proliferation of NSCLC. MARCH7 may be capable of regulating the activation and pathway molecules of the NF-κB and Wnt/β-catenin signaling pathways, in turn affecting the malignant proliferation of NSCLC cells.

The effects of MARCH7 on NSCLC *in vivo* were further explored. By constructing MARCH7 overexpression nude mouse tumor xenograft models, we studied the effects of MARCH7 on cell proliferation. The results showed that overexpression of MARCH7 was capable of accelerating the progression of cancerous tumors, and tumor volumes were also significantly higher than those of the control group. The expression of PCNA and Ki-67 in the MARCH7 overexpression group was visibly higher than that in the control group, as examined by immunohistochemistry analysis. Moreover, the MARCH7 overexpression group expressed higher levels of NF-κB p50, β-catenin and NF-κB p65 than the control group, which was in accordance with the results of the *in vivo* experiments. However, the exact biochemistry mechanism by which MARCH7 activates the NF-κB and Wnt/β-catenin signaling pathways is still unknown and needs further exploration.

Taken together, MARCH7 can promote the growth of tumors *in vivo*, and the molecular pathway through which MARCH7 is capable of regulating tumor cell proliferation *in vivo* is consistent with that in vitro. Given the effects of MARCH7 on NSCLC, MARCH7 could be identified as a potential target for NSCLC treatment and a novel option for patients with advanced NSCLC who have no indication for treatment and fail multiline chemotherapy. However, the sample size of this study was limited, and some exact mechanisms and signaling pathways are still unknown. Therefore, a larger sample size and further clinical research are needed to further confirm the conclusions above in the future.

References

1. Nasim F, Sabath BF, Eapen GA: Lung Cancer. The Medical clinics of North America 2019, 103(3):463-473.
2. Bade BC, Dela Cruz CS: Lung Cancer 2020: Epidemiology, Etiology, and Prevention. Clinics in chest medicine 2020, 41(1):1-24.
3. Mao Y, Yang D, He J, Krasna MJ: Epidemiology of Lung Cancer. Surgical oncology clinics of North America 2016, 25(3):439-445.
4. Jones GS, Baldwin DR: Recent advances in the management of lung cancer. Clinical medicine 2018, 18(Suppl 2):s41-s46.
5. Jonna S, Subramaniam DS: Molecular diagnostics and targeted therapies in non-small-cell lung cancer (NSCLC): an update. Discovery medicine 2019, 27(148):167-170.
6. Hoy H, Lynch T, Beck M: Surgical Treatment of Lung Cancer. Critical care nursing clinics of North America 2019, 31(3):303-313.
7. The L: Lung cancer: some progress, but still a lot more to do. Lancet 2019, 394(10212):1880.
8. Nagano T, Tachihara M, Nishimura Y: Molecular Mechanisms and Targeted Therapies Including Immunotherapy for Non-Small-Cell Lung Cancer. Current cancer drug targets 2019, 19(8):595-630.
9. Zhao K, Yang Y, Zhang G, Wang C, Wang D, Wu M, Mei Y: Regulation of the Mdm2-p53 pathway by the ubiquitin E3 ligase MARCH7. EMBO reports 2018, 19(2):305-319.
10. Liu L, Hu J, Yu T, You S, Zhang Y, Hu L: miR-27b-3p/MARCH7 regulates invasion and metastasis of endometrial cancer cells through Snail-mediated pathway. Acta biochimica et biophysica Sinica 2019, 51(5):492-500.
11. Hu J, Meng Y, Zeng J, Zeng B, Jiang X: Ubiquitin E3 Ligase MARCH7 promotes proliferation and invasion of cervical cancer cells through VAV2-RAC1-CDC42 pathway. Oncology letters 2018, 16(2):2312-2318.
12. Dolcet X, Llobet D, Pallares J, Matias-Guiu X: NF-kB in development and progression of human cancer. Virchows Archiv: an international journal of pathology 2005, 446(5):475-482.Sebio A, Kahn M, Lenz HJ: The potential of targeting Wnt/beta-catenin in colon cancer. Expert opinion on therapeutic targets 2014, 18(6):611-615.

13. Nguyen VHL, Hough R, Bernaudo S, Peng C: Wnt/beta-catenin signalling in ovarian cancer: Insights into its hyperactivation and function in tumorigenesis. Journal of ovarian research 2019, 12(1):122.
14. Lin S, Zhen Y, Guan Y, Yi H: Roles of Wnt/beta-Catenin Signaling Pathway Regulatory Long Non-Coding RNAs in the Pathogenesis of Non-Small-Cell Lung Cancer. Cancer management and research 2020, 12:4181-4191.
15. Qu J, Cheng T, Liu L, Heng J, Liu X, Sun Z, Wang W, Li K, Yang N: Mast cells induce epithelial-to-mesenchymal transition and migration in non-small-cell lung cancer through IL-8/Wnt/beta-catenin pathway. Journal of Cancer 2019, 10(22):5567.
16. Wang JY, Wang X, Wang XJ, Zheng BZ, Wang Y, Wang X, Liang B: Curcumin inhibits the growth via Wnt/beta-catenin pathway in non-small-cell lung cancer cells. European review for medical and pharmacological sciences 2018, 22(21):7492-7499.Nathan JA, Sengupta S, Wood SA, Admon A, Markson G, Sanderson C, Lehner PJ: The ubiquitin E3 ligase MARCH7 is differentially regulated by the deubiquitylating enzymes USP7 and USP9X. Traffic 2008, 9(7):1130-1145.
17. Zhao B, Ito K, Iyengar PV, Hirose S, Nakamura N: MARCH7 E3 ubiquitin ligase is highly expressed in developing spermatids of rats and its possible involvement in head and tail formation. Histochemistry and cell biology 2013, 139(3):447-460.
18. Zhang L, Wang H, Tian L, Li H: Expression of USP7 and MARCH7 Is Correlated with Poor Prognosis in Epithelial Ovarian Cancer. The Tohoku journal of experimental medicine 2016, 239(3):165-175.
19. Hu J, Zhang L, Mei Z, Jiang Y, Yi Y, Liu L, Meng Y, Zhou L, Zeng J, Wu H et al: Interaction of E3 Ubiquitin Ligase MARCH7 with Long Noncoding RNA MALAT1 and Autophagy-Related Protein ATG7 Promotes Autophagy and Invasion in Ovarian Cancer. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 2018, 47(2):654-666.
20. Wang JJ, Lei KF, Han F: Tumor microenvironment: recent advances in various cancer treatments. European review for medical and pharmacological sciences 2018, 22(12):3855-3864.
21. Saini J, Sharma PK: Clinical, Prognostic and Therapeutic Significance of Heat Shock Proteins in Cancer. Current drug targets 2018, 19(13):1478-1490.
22. Aoki MN, Amarante MK, de Oliveira CEC, Watanabe MAE: Biomarkers in Non-Small-Cell Lung Cancer: Perspectives of Individualized Targeted Therapy. Anticancer agents in medicinal chemistry 2018, 18(15):2070-2077.
23. Giustini NP, Jeong AR, Buturla J, Bazhenova L: Advances in Treatment of Locally Advanced or Metastatic Non-Small-Cell Lung Cancer: Targeted Therapy. Clinics in chest medicine 2020, 41(2):223-235.
24. Lu J, Han B: Liquid Biopsy Promotes Non-Small-Cell Lung Cancer Precision Therapy. Technology in cancer research & treatment 2018, 17:1533033818801809.
25. Giraud N, Abdiche S, Trouette R: Stereotactic radiotherapy in targeted therapy treated oligo-metastatic oncogene-addicted (non-small-cell) lung cancer. Cancer radiotherapie: journal de la Societe francaise de radiotherapie oncologique 2019, 23(4):346-354.

Figures

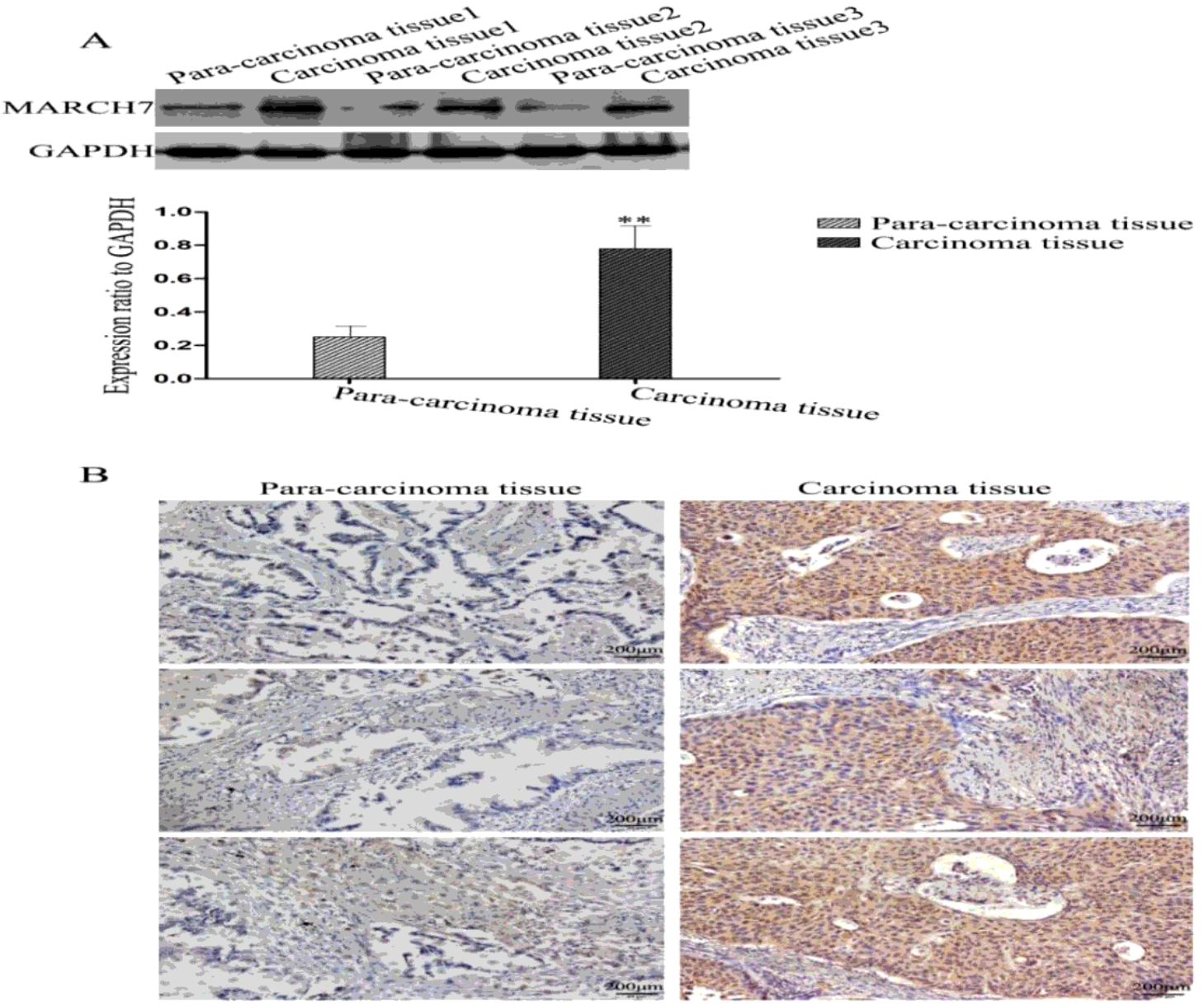


Figure 1

The Expression of MARCH7 in NSCLC

Notes: Expression Status of MARCH7 in NSCLC: (A) The differential MARCH7 expression between cancer tissues and adjacent tissues of NSCLC was detected by Western blot, and the gray value was recorded. (B) Immunohistochemistry was used to examine the expression status of MARCH7 in NSCLC. ** indicates that the difference was statistically significant, ** $p < 0.01$

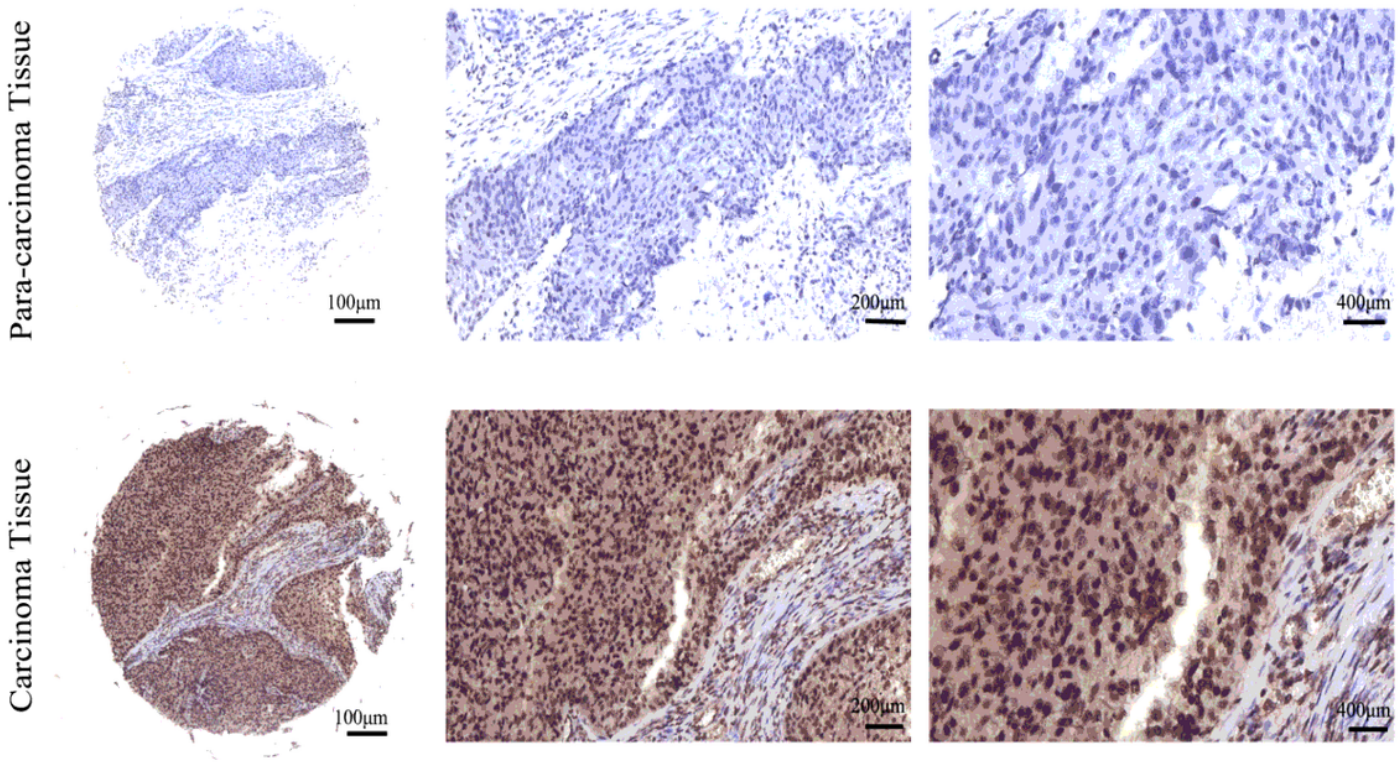


Figure 2

MARCH7 Expression in NSCLC and Adjacent Tissue Chips Assessed by Immunohistochemistry

Note: The images are the representative results of immunohistochemical staining of MARCH7.

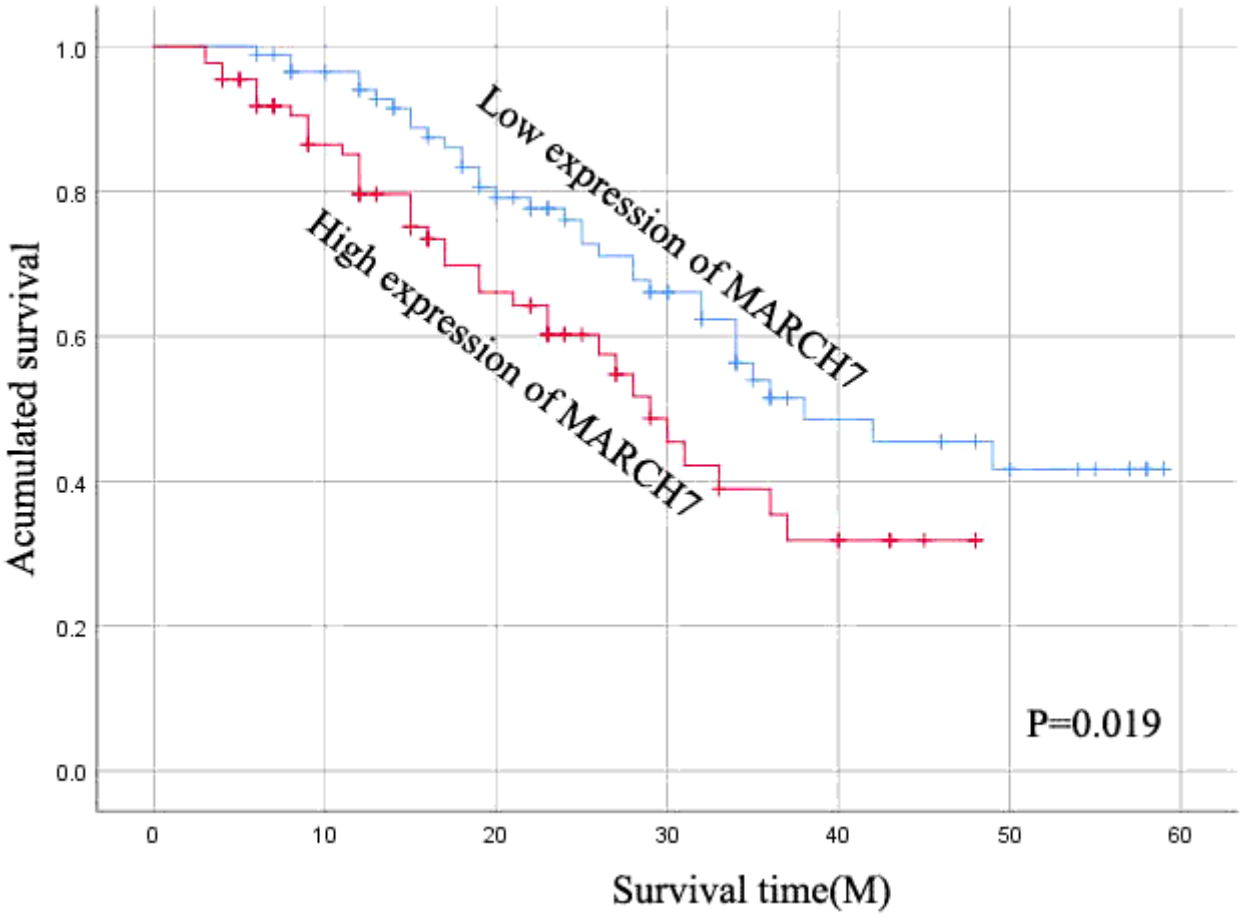


Figure 3

Kaplan–Meier Survival Curves

Note: Analysis of Kaplan–Meier Survival Curves in the High- and Low-Expression Group

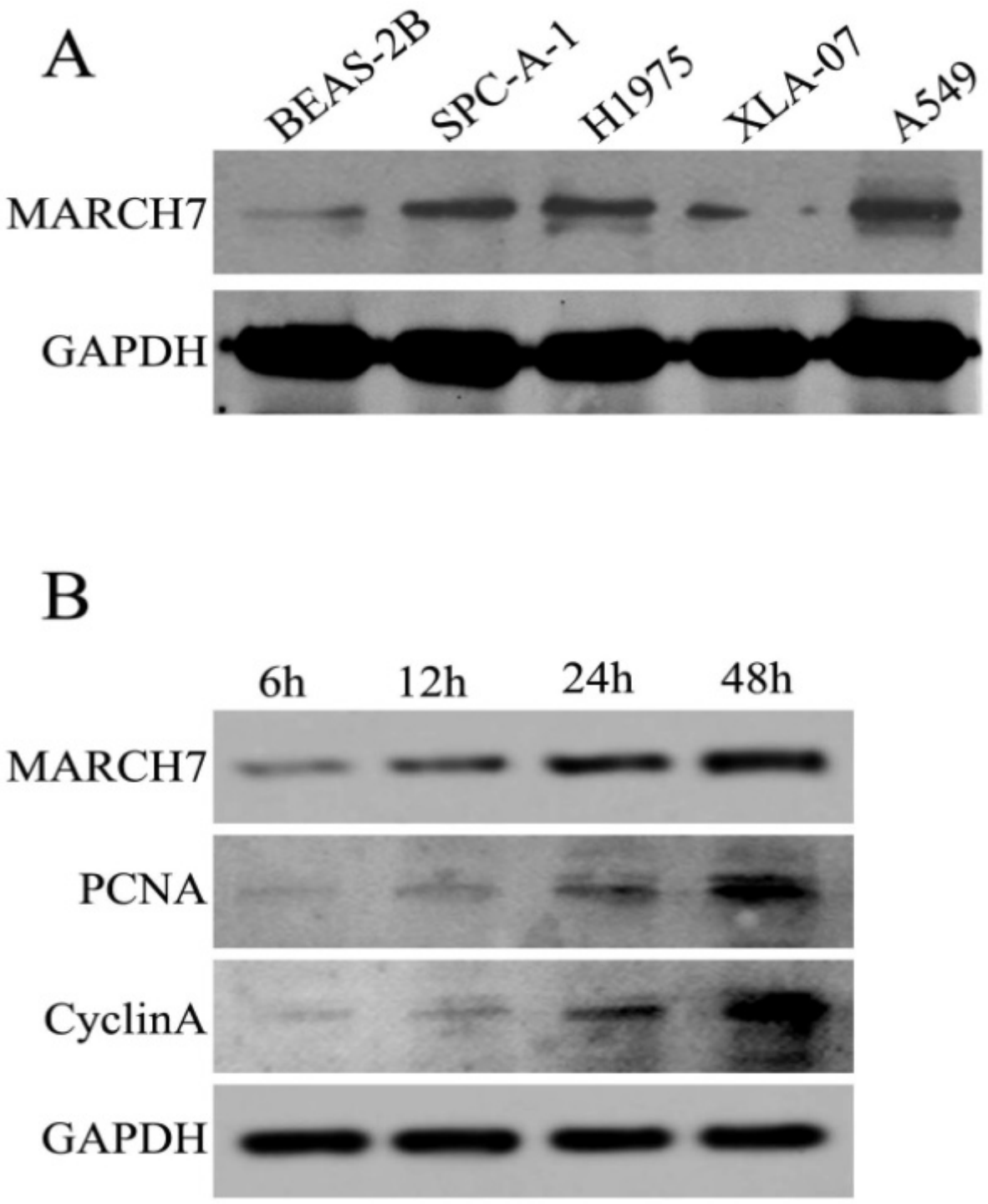
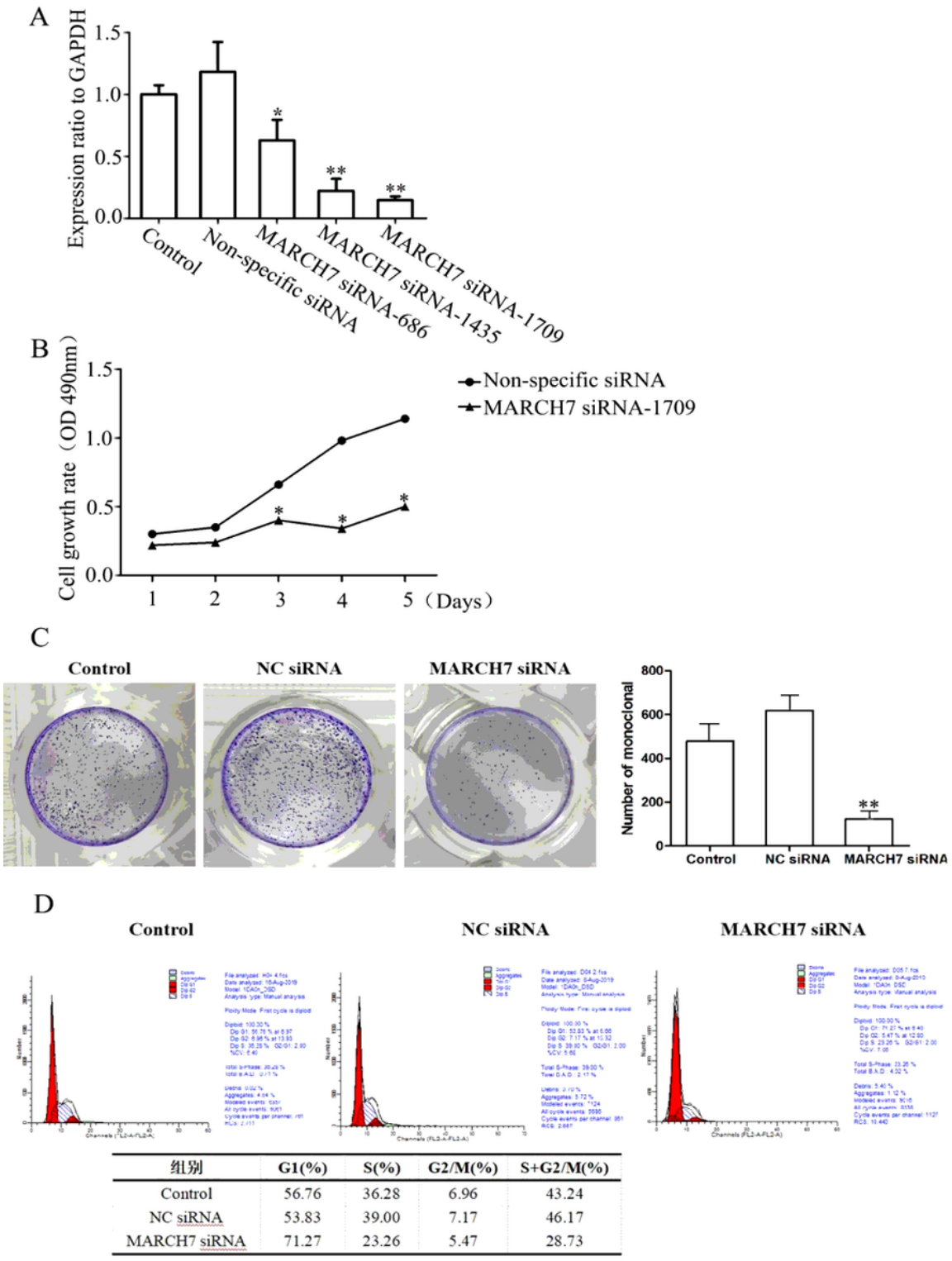


Figure 4

Association of MARCH7 Expression and Proliferation among NSCLC Cell Lines

Notes: (A) Western blot analysis was used to detect the level of MARCH7 expression in NSCLC cell lines. (B) Western blot analysis was used to detect the expression of MARCH7 at different time points and examine the expression of proliferation markers to preliminarily assess the relationship between MARCH7 and tumor cell proliferation.



组别	G1(%)	S(%)	G2/M(%)	S+G2/M(%)
Control	56.76	36.28	6.96	43.24
NC siRNA	53.83	39.00	7.17	46.17
MARCH7 siRNA	71.27	23.26	5.47	28.73

Figure 5

The Association between MARCH7 and Cell Proliferation

Notes: (A) MARCH7 siRNA was constructed, and siRNA efficiency was detected with qRT–PCR. (B) Cell proliferation was measured with CCK-8 after MARCH7 expression was altered. (C) The effect of intervening MARCH7 expression on cell proliferation was detected with a clone formation assay. (D) The

effect of intervening in MARCH7 expression on the cell cycle was detected with flow cytometry analysis. *indicates statistically significant difference p<0.05

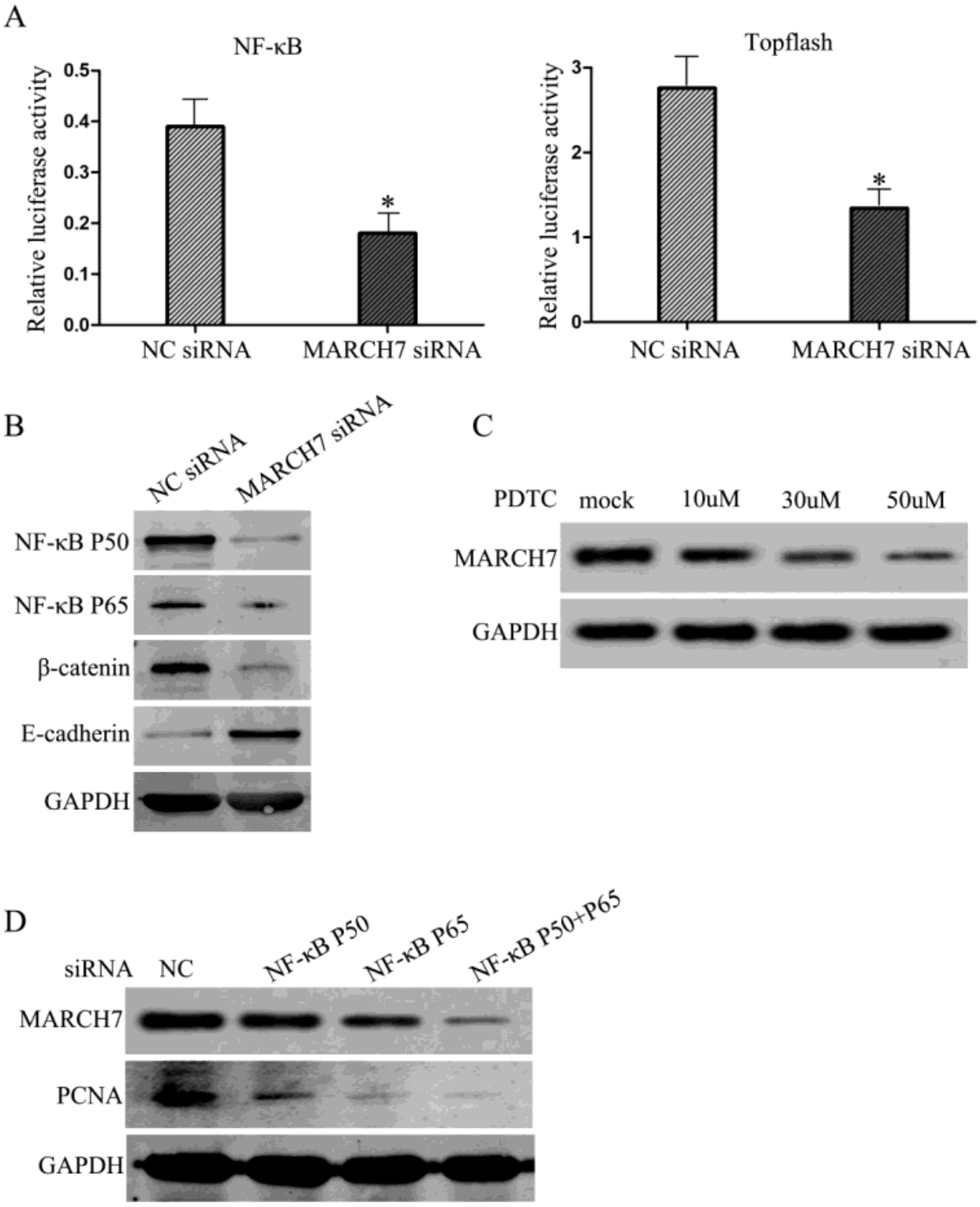


Figure 6

Investigation of the Molecular Mechanism of the Effects of MARCH7 on Cell Proliferation

Notes: (A) The effects of intervening in MARCH7 expression on the activity of NF-κB and the Wnt/β-catenin pathway were examined with a luciferase reporter gene assay. (B) The effects of intervening in MARCH7 expression on the activity of NF-κB and the Wnt/β-catenin signaling pathway were examined by Western blotting. (C) The effects of inhibiting the activity of the NF-κB signaling pathway on the expression of MARCH7 were examined by Western blotting. (D) The effects of intervening in the expression of the NF-κB signaling pathway on MARCH7 expression and cell proliferation were examined by Western blotting. *indicates statistically significant difference $p<0.05$

Figure 7

The Effects of MARCH7 Overexpression on the Tumor Size of Nude Mice

Notes: (A) The tumors were removed, and their size was recorded when the nude mouse tumorigenesis test was complete. (B) The effects of MARCH7 overexpression on tumor size were statistically analysed at different time points, and the tumor growth curve was plotted.

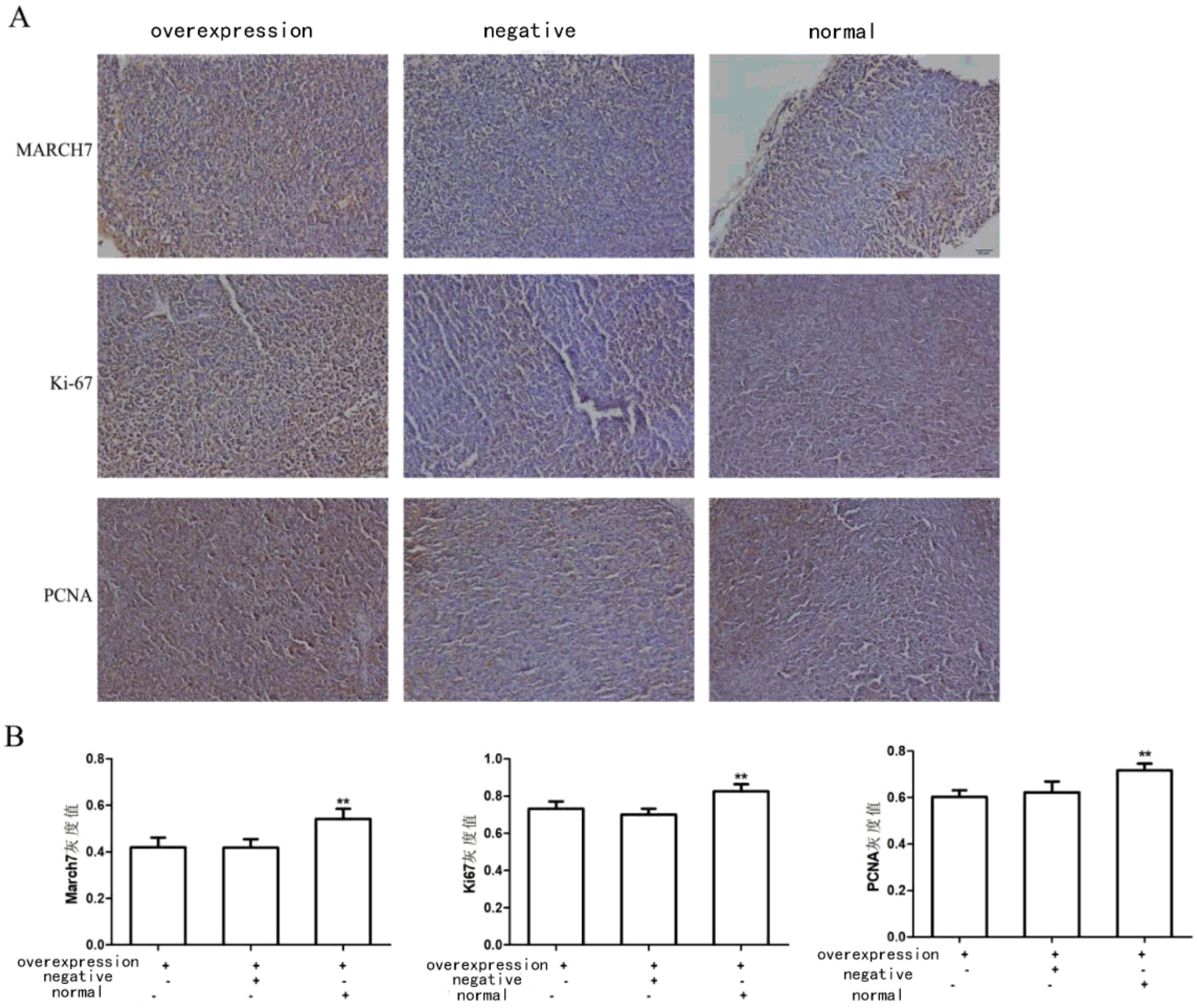


Figure 8

Expression and Cell proliferation of MARCH7 in Tumor Tissue

Notes: (A) Immunohistochemical analysis was performed on MARCH7 expression and cell proliferation of 3 groups of tumors in the nude mouse tumorigenesis test. (B) Statistical analysis was performed on the results of the immunohistochemical analysis above. *indicates a statistically significant difference $p<0.001$

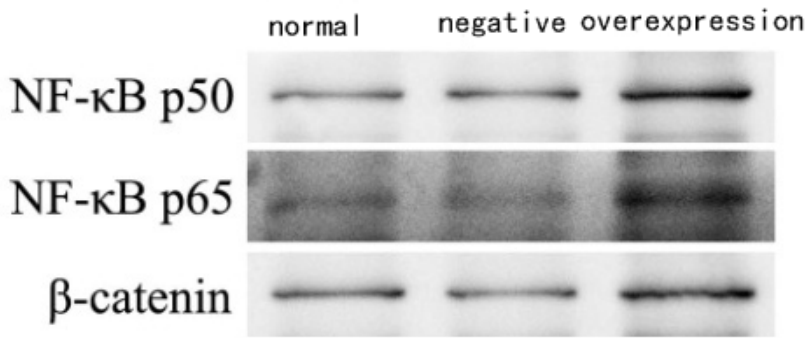


Figure 9

The Effects of MARCH7 Overexpression on the NF-κB and Wnt/β-catenin Signaling Pathways

Note: After Western blot analysis was performed on MARCH7-overexpressing cells, the expression of NF-κB and Wnt/β-catenin signaling pathway molecules was analysed, and gray value analysis was performed.

*indicates statistically significant difference $p<0.05$