Am J Transl Res 2017;9(3):1392-1401
www.ajtr.org /ISSN:1943-8141/AJTR0042825

Original Article

Nestin servers as a promising prognostic biomarker in non-small cell lung cancer

Fang Liu[1], Yuan Zhang[2], Ming Lu[3], Cong Wang[4], Qingbao Li[5], Yongsheng Gao[6], Dianbin Mu[6], Yan Cao[1], Miaomiao Li[1], Xiangjiao Meng[7]

Departments of [1]Imaging, [2]Laboratory Medicine, Shandong Medical College, Jinan 250002, Shandong, China; Departments of [3]Thoracic Surgery, [4]Radiation Oncology, Qilu Hospital of Shandong University, Jinan 250012, Shandong, China; [5]Department of Cardiac Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan 250021, Shandong, China; Departments of [6]Pathology, [7]Radiation Oncology, Shandong Cancer Hospital Affiliated to Shandong University, Shandong Academic of Medical Science, Jinan 250117, Shandong, China

Received October 20, 2016; Accepted March 6, 2017; Epub March 15, 2017; Published March 30, 2017

Abstract: Lung cancer is currently the leading cause of cancer-related death worldwide and it is important to identify the predictive and/or prognostic markers for the cancer. *Nestin*, a proliferative and multipotent biomarker has been reported to be associated with prognosis in non-small cell lung cancer (NSCLC) in a few studies. In the present study, we retrospectively recruited 153 patients with NSCLC. *Nestin* protein expression in tumor samples was determined by immunohistochemistry staining. *Nestin* expression was related with tumor differentiation (P=0.036), lymphatic metastasis (N stage, P=0.011), and p-TNM stage (P=0.013), while there was no significant association between *Nestin* expression level and age, smoking habits, gender, histologic type, and T stage. *Nestin* was an independent prognostic factor for overall survival in NSCLC with an adjusted hazard ratio of 2.701 (95% CI, 1.616-4.513, P<0.001) after controlling the confounding factors. Then we determined the effects of *Nestin* on cell proliferation, colony formation, invasion, and apoptosis by knockout of *Nestin* with a new developed method, CRISPR/Cas9 mediated genome editing. It was observed that knockout of *Nestin* caused enhancement of cancer cell apoptosis and inhibition of cell proliferation, colony formation, and invasion in A549 and H1299 cell lines. Furthermore, we examined the expression of epithelial-mesenchymal transition (EMT) related biomarkers such as E-cadherin and Vimentin in *Nestin*-depleted lung cancer cells and knockout of *Nestin* was found to inhibit EMT, suggesting the involvement of *Nestin* mediated EMT signaling in lung cancer. The finding above demonstrated that *Nestin* might serve as a prognostic factor and therapeutic target in NSCLCs.

Keywords: *Nestin*, non-small cell lung cancer, prognosis, proliferation, invasion

Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. The non-small cell lung cancer (NSCLC), approximately 80% of all lung cancer cases, are further divided into adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) [2]. Despite the advance of treatments including new chemotherapeutic agents introduction and surgical techniques improvement, the prognosis of NSCLC is still very low with only 15% patient survival at 5 years after diagnosis [3]. Thus the identification of predictive and/or prognostic markers is important to stratify patients with resected NSCLCs and to select high-risk patients who should receive aggressive adjuvant chemotherapy.

Nestin is a member of the intermediate filament (IF) family and serves as a potential proliferative and multipotent marker in progenitor and stem cells of the developing central nervous system (CNS) [4, 5]. It is expressed in the dividing cells of CNS and myogenic tissues during the early stages of development. Then its expression becomes rapidly downregulated and is replaced by tissue-specific intermediate filaments upon differentiation [6, 7]. In recent years, it is identified to be expressed in epithelial tumors and linked malignant characteristics and poor prognosis in pancreatic cancer, pros-

tate cancer, breast cancer, and lung cancer [8-12]. *Nestin* also has an anti-apoptotic function through inhibiting caspase activation [13].

In our latest research, we performed a systematic review and meta-analysis to clarify the correlations of *Nestin* expression with clinicopathologic features and prognosis in NSCLCs and found that *Nestin* high/positive expression was significantly associated with lymph node matastasis, TNM stage, and overall survival (OS) [14]. However, the included study number was very small (n=6) due to rare related studies, indicating more clinical evidence should be provided. Although a few in vitro studies have suggested that *Nestin* could promote cell proliferation in both NSCLC and small cell lung cancer (SCLC) cells and upregulated cell invasion in NSCLC cells, additional underlying mechanisms need to be investigated, especially for the effects of *Nestin* of cell invasion and migration [15-17].

Here, we retrospectively recruited 156 patients with NSCLC and determined the associations of *Nestin* expression with clinicopathologic features and OS. And we verified the role of *Nestin* in cell proliferation and invasion by knockout of *Nestin* in lung cancer cells with CRISPR/Cas9 mediated genome editing method. Furthermore, we have examined the regulation of epithelial-mesenchymal transition (EMT) related genes to reveal underlying mechanism of *Nestin* involved in cell invasion.

Materials and methods

Patients and tissue samples

156 patients with non-small cell lung cancer underwent surgery at Qilu Hospital of Shandong University in Jinan (China) and Shandong Cancer Hospital Affiliated to Shandong University from April 2000 to December 2010. After surgery, these patients were followed up for 3.2 to 109.3 months with a median follow up of 60.2 months. All patients were pathologically confirmed and their Formalin-Fixed and Parffin-Embedded (FFPE) cancer tissue samples were collected. The clinical characteristics of patients including age, sex, smoking habits, histologic grade, invasion depth (T stage), lymph node metastasis (N stage), distant metastasis (M stage), and differentiation were obtained from clinical or pathological records. The tumor, node, metastasis (TNM) classification was performed according to the American Joint Committee on Cancer staging manual (7th edition, 2010). The study protocol was approved by the ethics boards of Qilu Hospital of Shandong University and Shandong Cancer Hospital Affiliated to Shandong University, and tissue specimen acquisition was performed in accordance with the institutional guidelines.

Immunohistochemistry staining

All the FFPE cancer tissue specimens were serially sectioned at 5 μm in thickness. Tissue sections were deparaffinized in xylene and hydrated in graded ethanol. Then the sections were incubated in 3% hydrogen peroxide at room temperature for 5 to 10 minutes to eliminate endogenous peroxidase activity. After that they were heated in 10 mM citrate buffer (pH 6.0) for antigen retrieval. Goat serum was used to block the nonspecific binding. Then the slides were incubated with primary antibody (*Nestin*, 1:150, BD Biosciences) over night at 4°C followed by washing and incubation with secondary antibodies for 30 min at 37°C. The sections were next incubated with avidin-biotin complex for 60 min at 37°C and diaminobenzidine (DAB, ZLI-9032, ZSGB, Beijing, China) was added to develop peroxidase activity. PBS instead of the primary antibodies was used as negative controls. To evaluate the immunostaining intensity, the sections were observed under a light microscope and scored by three independent investigators. Conflicting scores were resolved by selecting the value that was consistent between two observers or the average of the scores. The final score was determined by multiplying the staining intensity (scored as: 0, no staining; 1, weak staining; 2, moderate staining and 3, strong staining) by the percentage of positive cells (scored as: 0, 0-10% positive cells; 1, 10-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, 76-100% positive cells). The expression of *Nestin* was divided into low expression group and high expression group by the cut-off value (final score, 4) obtained from X-title software according clinical outcomes [18].

Cell culture

Human A549 and H1299 cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Knockout of Nestin gene by CRISPR/Cas9-mediated genome editing

Target sequences for CRISPR interference were designed at CRISPR direct (http://crispr.dbcls.jp/) which was provided by the Database Center for Life Science (Chiba, Japan). The target sequences for human *Nestin* are Sg1+: CCTACAGAGCCAGATCGCTC. Two complementary oligonucleotides with BpiI restriction sites for guide RNAs (gRNAs) were synthesized and cloned into pX459 CRISPR/Cas9-Puro vector (Addgene, Cambridge, MA) deposited by the Feng Zhang Lab. A549 or H1299 cells were transfected with pX459-gRNA using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After transfection, these cells were selected with 2 μg/ml of puromycin for two days and then reseeded at 500 cells per well of a 96-well plate. Expressions of *Nestin* of the expanded colonies were detected by immunoblotting to select the *Nestin* knockout colonies. The genome sequences of the edited locus in selected colonies were confirmed by sequencing.

Western blotting

Cells were lysed by RIPA Lysis Buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, sodium orthovanadate, sodium fluoride, EDTA, leupeptin) and PMSF (Phenylmethanesulfonyl fluoride, Beyotime, China) and proteins were extracted. The extracts were separated by SDS-PAGE gel electrophoresis, transferred to polyvinylidenedifluoride membrane, blocked with 5% non-fat dry milk, and incubated with primary antibodies (*Nestin*, 1:1000, BD Biosciences; vimentin and E-cadherin, 1:1000, DAKO; β-actin antibody, 1:1000, Abcam, Cambridge, MA, USA) overnight at 4°C. Then, horseradish peroxidase-conjugated secondary antibodies were added. Bands were subsequently visualized using a chemiluminescence detection system (EMD Millipore, Billerica, MA, USA) and density was determined using an image analyzer.

CCK8 assay

The proliferation of A549 and H1299 cells was detected using 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2Htetrazolium monosodium salt (cell counting kit-8 (CCK8)). After knockout of *Nestin*, the cells were seeded in 96-well plates at a cell density of 5 × 10^4/well and incubated for 0, 24, 48, and 72 h. At different time intervals, the cells were incubated with CCK8 reagent for 1 h at 37°C. The absorbance of each well was measured at 450 nm using Thermo Scientific Varioskan Flash (Thermo Scientific, Finland) and percentage of viable cells were calculated.

Clonogenic assay

Nestin depleted A549 or H1299 cells were treated with Trypsin to generate a single cell suspension and seeded in 6-well plates at 500 cells per well. 14 days after seeding, colonies were stained with crystal violet, and the number of colonies containing at least 50 cells was counted and the colony survival fraction was calculated.

Transwell invasion assay

The transwell membrane (8-mm pore size, 6.5-mmdiameter, Corning Costar) was coated with Matrigel (BD Biosciences). *Nestin*-depleted A549 or H1299 cells were resuspended in serum-free medium and added to the upper chamber of precoated transwells at a density of 2.0 × 10^5/ml. The lower chamber contained normal medium with 10% FBS. After incubation for 24 h, a cottontipped swab was used to swab the cells on the upper chamber. The invasive cells, which were attached to the lower surface of the membrane, were fixed with methanol and stained with 0.1% Crystal violet (Sigma). Then the number of the invasive cells (5 fields per filter) was counted under an inverted microscope.

Apoptosis analysis by FACS

A549 and H1299 cells were plated at a density of 1 × 10^6 cells/well in six-well plates overnight. Media were removed and cells were incubated with CRISPR-Nestin at a multiplicity of infection of 30 of A549 or MOI=10 of H1299, or control with PBS in 5 ml media for 2 h, after which 10 ml media was added. After 72 h, harvested cells were trypsinized, washed with PBS and apoptosis analysis was performed with the Annexin V-FITC apoptosis detection kit and then analyzed by flow cytometry (both from BD Biosciences, San Jose, CA, USA).

Table 1. Basal characteristics of the patients and correlations of *Nestin* with clinicopathologic features

Clinical features	Total	Nestin expression		*P*-value
		Low	High	
Age				0.128
<60	83	60	23	
≥60	70	58	12	
Smoke				0.562
Yes	90	71	19	
No	63	47	16	
Gender				0.677
Male	106	83	23	
Female	47	35	12	
Histology				0.918
ADC	66	51	15	
SCC	64	50	14	
Others	23	17	6	
Differentiation				0.036
Poor	47	39	8	
Moderate	85	60	25	
Well	21	19	1	
T stage				0.098
T1	39	34	5	
T2	90	65	25	
T3	13	12	1	
T4	11	7	4	
N stage				0.011
N0	94	78	16	
N1	25	21	4	
N2	32	18	14	
N3	2	1	1	
p-TNM stage				0.013
I	74	62	12	
II	35	29	6	
III	44	27	17	
Outcome				<0.001
Alive	81	74	7	
Death	68	40	28	
Lost follow-up	4	4	0	

Statistical analysis

All statistical analyses were performed with the SPSS 19.0 software (SPSS, Chicago, IL). Correlations between *Nestin* and clinicopathological factors were evaluated by the chi-square test. The Kaplan-Meier method was used to calculate the survival curves, and the log-rank test was used to compare the survival difference between patient subgroups according to *Nestin* expression. Univariate and multivariate Cox regression analyses were performed to investigate the prognostic value of *Nestin* expression in overall survival. Difference of CCK8 index, colony formation, apoptosis, and cell invasion between *Nestin*-depleted group and control group were compared with Student's t-test. Pearson and Spearman tests were performed to analyze the mRNA correlations of Nestin with EMT biomarkers in database TCGA and correlation coefficient r was used to indicate correlation degrees. Two-sided *P* value <0.05 was considered statistically significant.

Results

Patient characteristics

The clinicopathologic characteristics of 153 patients were summarized in **Table 1**. Of the patients, 106 were male and 47 were female with a median age of 59 (29-88) years. 90 patients had smoking habit. There were 66 ADC, 64 SCC, and 23 other NSCLCs including mixed type of ADC and SCC, large cell neuroendocrine carcinomas, and large cell carcinomas. 21 cases were well-differentiated, 85 cases were moderate-differentiated, and 47 cases were poor-differentiated. According to TNM classification, the patients with stage I, II, and III NSCLCs were 74, 35, and 44, respectively. The overall follow-up durations ranged from 3.2 to 109.3 months (median, 62 months). A total of 81 patients were alive at the end of the follow-up, 68 patients died of lung cancer, and four patients were lost to follow-up.

Correlations of Nestin expression with clinicopathologic characteristics

Nestin expression in tumor samples were determined by immunohistochemistry, the staining intensity and the percentage of positive cells were evaluated and their products were used to indicate *Nestin* expression (**Figure 1**). With the aid of the software X-title, the cut-off value of *Nestin* expression was calculated according to the clinical outcomes and the expression of *Nestin* was divided into low expression group and high expression group. 118 patients were *Nestin* low expression and 35 patients were high expression. Then the correlations of *Nestin* expression with clinicopathological characteristics were analyzed. As sh-

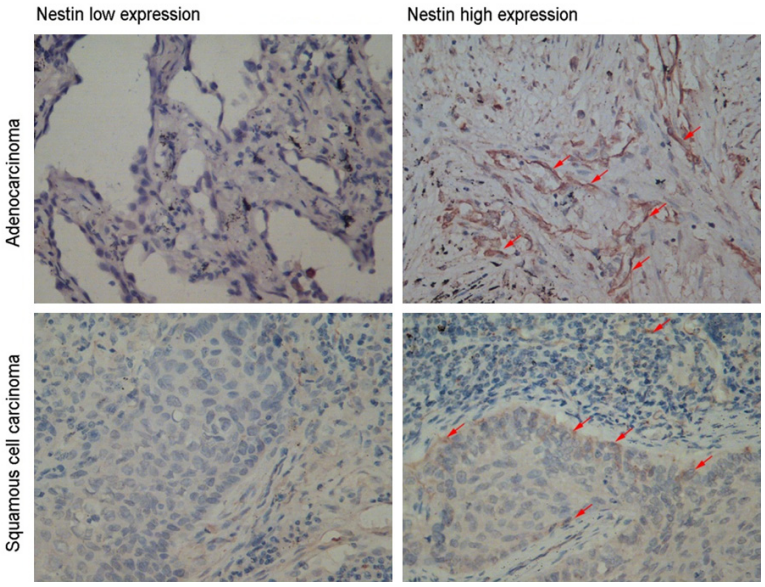

Figure 1. Immunohistochemical stain analysis of *Nestin* expression in non-small cell lung cancer (NSCLC, 400 ×). Note: Arrow, *Nestin* expression.

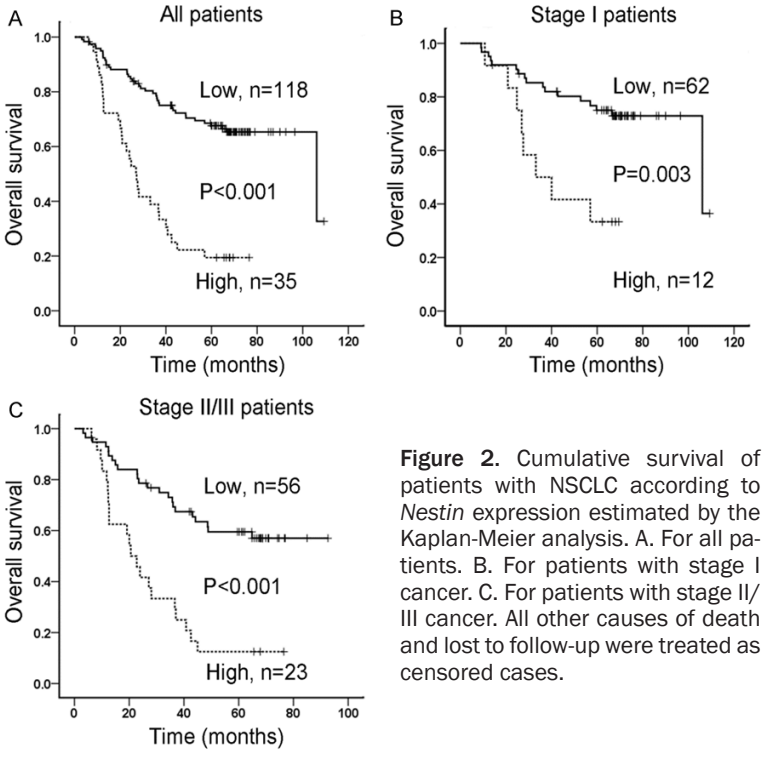

Figure 2. Cumulative survival of patients with NSCLC according to *Nestin* expression estimated by the Kaplan-Meier analysis. A. For all patients. B. For patients with stage I cancer. C. For patients with stage II/III cancer. All other causes of death and lost to follow-up were treated as censored cases.

own in **Table 1**, *Nestin* expression was related with tumor differentiation (P=0.036), lymphatic metastasis (N stage, P=0.011), and p-TNM stage (P=0.013), while it did not correlated with age, smoking habits, gender, histological cancer type, and T stage.

High Nestin expression was associated with poor prognosis in NSCLC patients

To investigate the effects of *Nestin* expression on prognosis in NSCLC patients, Kaplan-Meier analysis was firstly performed. As shown in **Figure 2**, the cumulative overall survival probability of the patients with high *Nestin* expression was lower than those with low *Nestin* expression in whole cohort (P<0.001, **Figure 2A**). Furthermore, the association of high *Nestin* expression with poor prognosis was also observed in the patients with stage I cancers or stage II/III cancers (**Figure 2B**, **2C**). Then, a Cox proportional hazards model was applied to estimate the effect of *Nestin* expression on overall survival. *Nestin* high expression increased the hazard of lung cancer related death by six times than that of *Nestin* low expression (HR, 6.069; 95% CI, 2.805-13.132; P<0.001, **Table 2**). In addition, the clinicopathologic features including smoking habits, differentiation, T stage, and N stage were significantly associated with overall survival in NSCLC in Univariate analysis. After controlling these clinicopathological features, the adjusted HR of *Nestin* high expression was 2.701 (95% CI, 1.615-4.516) compared with *Nestin* low expression, suggesting *Nestin* was an independent risk factor for poorer survival.

Knockout of Nestin altered cancer cell proliferation, invasion, and apoptosis

To reveal the role of *Nestin* in lung cancer, we used CRISPR/Cas9 directed gene editing to knockout *Nestin* expression in human lung cancer cell lines, A549 and H1299, and deter-

Table 2. Univariate and multivariate analyses of *Nestin* in prognosis of NSCLCs

Parameters	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.113	0.694-1.785	0.658			
Smoke	0.616	0.382-0.992	0.046	1.822	1.093-3.039	0.022
Gender	0.760	0.462-1.249	0.279			
Histology	1.013	0.727-1.411	0.939			
Differentiation	3.534	1.282-9.709	0.015	3.097	1.101-8.711	0.032
T stage	1.968	1.055-3.673	0.033	1.796	0.942-3.425	0.075
N stage	2.455	1.521-3.962	<0.001	1.981	1.215-3.228	0.006
Nestin	6.069	2.805-13.132	<0.001	2.701	1.616-4.513	<0.001

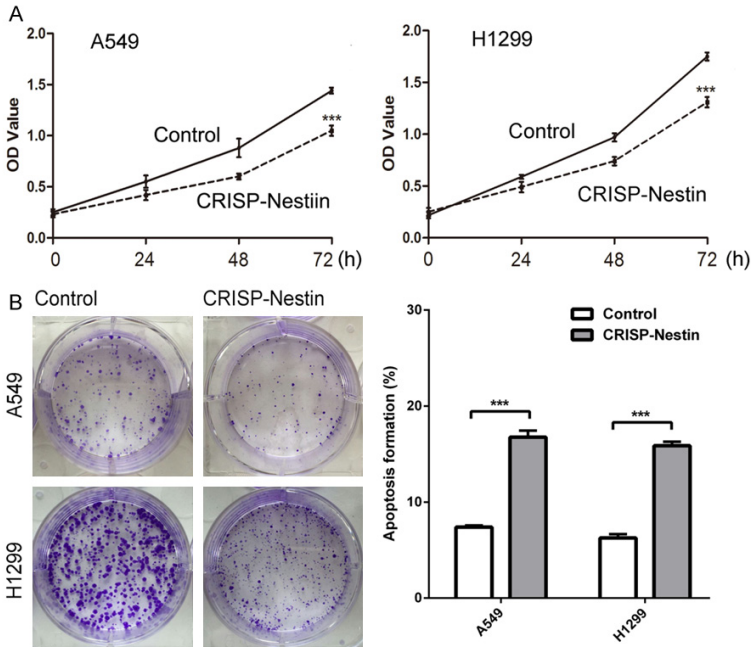

Figure 3. Effect of *Nestin* on lung cancer cell growth and colony formation. A. Cell growth after *Nestin* depletion was analyzed by CCK8 assay in A549 and H1299 cells. B. Cell colony formation after *Nestin* depletion was analyzed by crystal violet staining. *, **, and ***indicate P<0.05, P<0.01, and P<0.001, respectively.

mined the effects of *Nestin* on cell proliferation, apoptosis, and invasion. In the CCK8 assays, knockout of *Nestin* inhibited cell growth in both A549 and H1299 cells (**Figure 3A**). Knockout of *Nestin* also resulted in prominent decrease in colony-forming ability and invasion in A549 and H1299 cells (colony formation, 0.43% to 0.20% and 0.52% to 0.27%; invasion, 167.00 to 63.00 and 120.67 to 61.67; **Figures 3B** and **4A**). On the other hand, an increase in cell apoptosis was observed in *Nestin*-depleted A549 and H1299 cells (7.40% to 16.77% and 6.27% to 15.87%, **Figure 4B**). The results were consistent with our clinical outcomes that high expression of *Nestin* was associated with poor prognosis in NSCLC and the previous reports. Given that a few studies have explored the underlying mechanism of *Nestin* regulating cell proliferation, here, we focused how *Nestin* modulated cell invasion in NSCLC cells.

We hypothesized that *Nestin* might promote cell invasion via regulating the expression of epithelial-mesenchymal transition (EMT) pathway related genes. Then we examined the effects of *Nestin* on EMT related biomarkers in *Nestin* depleted cells and the results suggested that, after knockout of *Nestin*, E-cadherin expression was increased whereas Vimentin expression was inhibited (**Figure 4C**). To verify the conclusion, *Nestin* and EMT biomarkers mRNA expression profile from 1124 patients with NSCLCs were downloaded from online database, TCGA, and association analysis suggested *Nestin* was correlated with some EMT biomarkers, especially Vimentin and transcription factors, Zeb1 and ETS1 (**Table 3**).

Discussion

In the present study, we analyzed the correlation of *Nestin* expression with clinicopathological features and overall survival in 156 resected NSCLC patients. The results demonstrated that *Nestin* expression was related with tumor differentiation, lymphatic metastasis. Survival analysis showed that high *Nestin* expression was an independent prognostic factor for poor survival in NSCLC. To investigate the underlying mechanisms of *Nestin* involved in NSCLC, we also revealed the effects of *Nestin* on cell prolif-

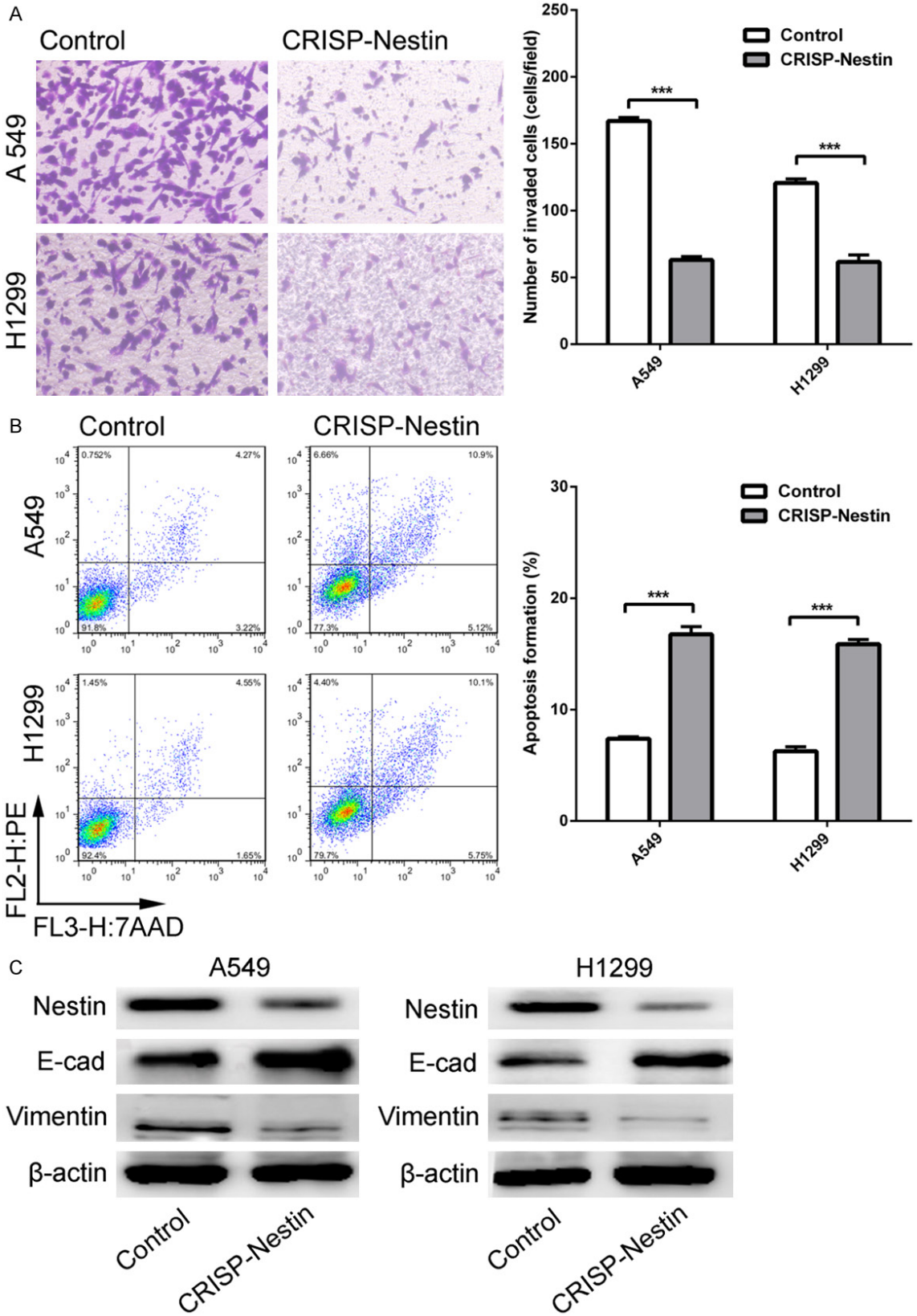

Figure 4. Effects of *Nestin* on cell apoptosis, invasion, and expression of biomarkers related to epithelial-mesenchymal transition (EMT) in lung cancer cells. Cell apoptosis (A) and cell invasion (B) were analyzed by FACS and transwell assay, respectively, after knockout of Nestin in A549 and H1299 cells. (C) Expression of Nestin and EMT biomarkers, E-cad and Vimentin, in Nestin-depleted A549 and H1299 cells were examined by western blotting. ***indicate P<0.001.

Table 3. Correlation of *Nestin* with EMT biomarkers at the transcription level in NSCLC from TCGA database

EMT biomarkers	Pearson		Spearman	
	r	*P*-value	r	*P*-value
Claudin	-0.2885	<0.001	-0.2799	<0.001
Twist1	-0.1586	<0.001	-0.1667	<0.001
E-cad	-0.1557	<0.001	-0.1874	<0.001
Slug	-0.08256	0.006	-0.08302	0.005
LEF1	-0.04169	0.163	-0.04387	0.142
β-catenin	0.1195	<0.001	0.1249	<0.001
N-cad	0.1253	<0.001	0.1344	<0.001
Occludin	0.1851	<0.001	0.2106	<0.001
α-catenin	0.2573	<0.001	0.261	<0.001
Snail	0.3572	<0.001	0.3466	<0.001
ZO-1	0.3879	<0.001	0.3574	<0.001
Vim	0.5057	<0.001	0.5129	<0.001
Zeb1	0.5244	<0.001	0.5007	<0.001
ETS1	0.5278	<0.001	0.5243	<0.001

eration, apoptosis, and invasion by knockout of *Nestin* with CRISPR/Cas9 method.

Up to now, there were only six studies that explored the potential correlations of *Nestin* protein expression with clinicopathologic parameters and prognosis in NSCLC. We summarized these studies in a recent meta-analysis and found Nestin high/positive expression was significantly associated with lymph node matastasis, TNM stage, and overall survival (OS) in NSCLC [14]. Another meta-analysis also revealed that the associations of *Nestin* protein expression with lymph node metastasis and TNM stage in regardless of cancer types [19]. Consistent with these results, we also identified the associations between *Nestin* expression and lymphatic metastasis and p-TNM stage in NSCLC. For the overall survival, subgroups stratified by p-TNM stage were also performed and the results among patients with stage I cancer and stage II-IV cancer were consistent.

For the molecular function of *Nestin* in lung cancer, we found that knockout of *Nestin* by CRISPR inhibited cell proliferation, colony formation ability, and invasion in lung cancer cell lines A549 and H1299, the results were similar with the previous reports in NSCLC cells in vitro [15-17]. Further, we also determined the effects of *Nestin* on cell apoptosis and this was the first time to report the apoptosis increase after downregulation of *Nestin* in NSCLC. A few studies have demonstrated some underlying mechanisms of *Nestin* in regulating lung cancer cell phenotype. Chen et al. reported that *Nestin* can promote AKT-GSK3b-Rb signaling pathway to increase cell cycle progression phosphorylation allowing the expression of genes necessary to enter the S phase in cell cycle [15]. In this pathway, *Nestin* aberrant expression increases AKT phosphorylation leading to the phosphorylation of GSK3b and Rb [20-22]. In the present study, to reveal the mechanisms of *Nestin* contributing to cell invasion, we evaluated the change of epithelial mesenchymal transition (EMT) related biomarkers, E-cadherin and Vimentin after *Nestin* depletion and found that *Nestin* depletion increased E-cadherin expression while inhibited Vimentin, suggesting *Nestin* modulated EMT to promote cell invasion, resulting in lung cancer tumor metastasis. The analysis of the TCGA mRNA profile consisting of 1124 NSCLC cancer also suggested that levels of *Nestin* mRNA expression were correlated with the changes of the mRNA expression of EMT biomarkers. In addition, *Nestin* was known as one of the marker for cancer stem cells (CSCs), which were defined as a subpopulation that can self-renew and maintain than tumor presence and resistant to radiation and chemotherapy [23-25]. Regard of the involvement of *Nestin* in EMT in lung cancer, it might maintain cancer stem cell state partly via inducing continuous EMT signals, consistent with the recent findings in Anaplastic thyroid carcinoma [26].

Currently, little is known regarding the mechanism of expression of *Nestin* in cancers, especially in lung cancer. It has been reported that the in vivo transcription of *Nestin* is upregulated by the thyroid transcription factor gene 1 (TTF-1), which plays a significant role in the development of small cell lung cancer [27, 28]. In addition, Watkins et al. have reported that *Nestin* was decreased by inhibition of Hedgehog (Hh) signaling in an SCLC cell line and this signaling known as crucial for the regulation of NE features and the survival of SCLC [29, 30]. And Narita et al. found that inhibition of Akt and p-Akt by Akt inhibitor IV resulted in downregulation of *Nestin* in lung adenocarcinoma cells [17].

Some shortcomings still existed in our study. Firstly, prognostic value of Nestin in lung cancer should be estimated in more clinical sample and research centers. Secondly, all though we verified the effects of Nestin on EMT biomarkers, E-cad and Vim, in vitro and the correlations of Nestin with EMT biomarkers in 1124 patients mRNA profile from TCGA database, detailed mechanisms that how Nestin modulated EMT biomarker expression had not been investigated. Thirdly, as Nestin was a biomarker of CSCs, crosstalk between the signaling of CSC and EMT might be involved in the development in lung cancer and should be explored in the future.

In summary, we found that *Nestin* was an independent prognostic factor for overall survival in NSCLC after retrospective analysis of 156 patients underwent surgery. In vitro experiments suggested that knockout of Nestin with CRISPR/Cas9 mediated genome editing caused enhancement of cancer cell apoptosis and inhibition of cell proliferation, colony formation, and invasion in lung cancer cells. Furthermore, mechanism research revealed that *Nestin* might regulate that cell process by mediating EMT signaling in lung cancer. All the evident above demonstrated that *Nestin* might serve as a prognostic factor and therapeutic target in NSCLCs.

Acknowledgements

This study was supported by the grants from National Natural Science Foundation of China (No.81301868) and Higher Educational Science and Technology Program of Shandong Province (No.J15LK04).

Disclosure conflict of interest

None.

Authors' contribution

Designed the study: F. L., Y. Z., and X. M. Molecular and cellular experiments: F. L., Y. Z., C. W., Q. L., and M. L. Statistical analyses: F. L., C. W., Q. L., Y. C., and M. L. Pathology and IHC analysis: Y. G. and D. M. Wrote the main manuscript text: F. L. and X. M. All authors reviewed the manuscript.

Address correspondence to: Xiangjiao Meng, Department of Radiation Oncology, Shandong Cancer Hospital Affiliated to Shandong University, Shandong Academic of Medical Science, No. 440, Jiyan Road, Jinan 250117, Shandong, China. Tel: +86-531-67626995; Fax: +86-531-67626819; E-mail: sdxjmeng@163.com

References

[1] Torre LA, Siegel RL and Jemal A. Lung cancer statistics. Adv Exp Med Biol 2016; 893: 1-19.

[2] Travis WD. Pathology of lung cancer. Clin Chest Med 2011; 32: 669-692.

[3] D'Addario G, Felip E; ESMO Guidelines Working Group. Non-small-cell lung cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. Ann Oncol 2009; 20 Suppl 4: 68-70.

[4] Park D, Xiang AP, Mao FF, Zhang L, Di CG, Liu XM, Shao Y, Ma BF, Lee JH, Ha KS, Walton N and Lahn BT. Nestin is required for the proper self-renewal of neural stem cells. Stem Cells 2010; 28: 2162-2171.

[5] Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G and Hoffman RM. Nestin expression in hair follicle sheath progenitor cells. Proc Natl Acad Sci U S A 2003; 100: 9958-9961.

[6] Lendahl U, Zimmerman LB and McKay RD. CNS stem cells express a new class of intermediate filament protein. Cell 1990; 60: 585-595.

[7] Bieberich E, MacKinnon S, Silva J, Noggle S and Condie BG. Regulation of cell death in mitotic neural progenitor cells by asymmetric distribution of prostate apoptosis response 4 (PAR-4) and simultaneous elevation of endogenous ceramide. J Cell Biol 2003; 162: 469-479.

[8] Chen Z, Wang T, Luo H, Lai Y, Yang X, Li F, Lei Y, Su C, Zhang X, Lahn BT and Xiang AP. Expression of nestin in lymph node metastasis and lymphangiogenesis in non-small cell lung cancer patients. Hum Pathol 2010; 41: 737-744.

[9] Wu B, Sun C, Feng F, Ge M and Xia L. Do relevant markers of cancer stem cells CD133 and Nestin indicate a poor prognosis in glioma patients? A systematic review and meta-analysis. J Exp Clin Cancer Res 2015; 34: 44.

[10] Shan YS, Chen YL, Lai MD and Hsu HP. Nestin predicts a favorable prognosis in early ampullary adenocarcinoma and functions as a promoter of metastasis in advanced cancer. Oncol Rep 2015; 33: 40-48.

[11] Kurata K, Onoda N, Noda S, Kashiwagi S, Asano Y, Kawajiri H, Takashima T, Tanaka S, Ohsawa M and Hirakawa K. Nestin expression as an independent indicator of poor prognosis

for patients with anaplastic thyroid cancer. Oncol Lett 2015; 10: 850-856.
[12] Tabata K, Matsumoto K, Minami S, Ishii D, Nishi M, Fujita T, Saegusa M, Sato Y and Iwamura M. Nestin is an independent predictor of cancer-specific survival after radical cystectomy in patients with urothelial carcinoma of the bladder. PLoS One 2014; 9: e91548.
[13] Huang YL, Wu CM, Shi GY, Wu GC, Lee H, Jiang MJ, Wu HL and Yang HY. Nestin serves as a prosurvival determinant that is linked to the cytoprotective effect of epidermal growth factor in rat vascular smooth muscle cells. J Biochem 2009; 146: 307-315.
[14] Li Q, Liu F, Zhang Y, Fu L, Wang C, Chen X, Guan S and Meng X. Association of SOX2 and Nestin DNA amplification and protein expression with clinical features and overall survival in non-small cell lung cancer: a systematic review and meta-analysis. Oncotarget 2016; 7: 34520-31.
[15] Chen Z, Wang J, Cai L, Zhong B, Luo H, Hao Y, Yu W, Wang B, Su C, Lei Y, Bella AE, Xiang AP and Wang T. Role of the stem cell-associated intermediate filament nestin in malignant proliferation of non-small cell lung cancer. PLoS One 2014; 9: e85584.
[16] Takakuwa O, Maeno K, Kunii E, Ozasa H, Hijikata H, Uemura T, Kasai D, Ohkubo H, Miyazaki M, Oguri T and Niimi A. Involvement of intermediate filament nestin in cell growth of small-cell lung cancer. Lung Cancer 2013; 81: 174-179.
[17] Narita K, Matsuda Y, Seike M, Naito Z, Gemma A and Ishiwata T. Nestin regulates proliferation, migration, invasion and stemness of lung adenocarcinoma. Int J Oncol 2014; 44: 1118-1130.
[18] Zhang JX, Cai MB, Wang XP, Duan LP, Shao Q, Tong ZT, Liao DZ, Li YY, Huang MY, Zeng YX and Shao JY. Elevated DLL4 expression is correlated with VEGF and predicts poor prognosis of nasopharyngeal carcinoma. Med Oncol 2013; 30: 390.
[19] Zhong B, Wang T, Zou J, Zheng F, Huang R, Zheng X, Yang W and Chen Z. Association of the intermediate filament nestin with cancer stage: a meta-analysis based on 223 positive/high nestin cases and 460 negative/low case-free controls. Oncotarget 2015; 6: 22970-22977.
[20] Diehl JA, Cheng M, Roussel MF and Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998; 12: 3499-3511.
[21] Etienne-Manneville S and Hall A. Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature 2003; 421: 753-756.
[22] Turjanski AG, Vaque JP and Gutkind JS. MAP kinases and the control of nuclear events. Oncogene 2007; 26: 3240-3253.
[23] Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL and Wahl GM. Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 2006; 66: 9339-9344.
[24] Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C and De Maria R. Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 2008; 15: 504-514.
[25] Reya T, Morrison SJ, Clarke MF and Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414: 105-111.
[26] Liu J and Brown RE. Immunohistochemical detection of epithelialmesenchymal transition associated with stemness phenotype in anaplastic thyroid carcinoma. Int J Clin Exp Pathol 2010; 3: 755-762.
[27] Pelizzoli R, Tacchetti C, Luzzi P, Strangio A, Bellese G, Zappia E and Guazzi S. TTF-1/NKX2.1 up-regulates the in vivo transcription of nestin. Int J Dev Biol 2008; 52: 55-62.
[28] Kitamura H, Yazawa T, Sato H, Okudela K and Shimoyamada H. Small cell lung cancer: significance of RB alterations and TTF-1 expression in its carcinogenesis, phenotype, and biology. Endocr Pathol 2009; 20: 101-107.
[29] Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA and Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature 2003; 422: 313-317.
[30] Park KS, Martelotto LG, Peifer M, Sos ML, Karnezis AN, Mahjoub MR, Bernard K, Conklin JF, Szczepny A, Yuan J, Guo R, Ospina B, Falzon J, Bennett S, Brown TJ, Markovic A, Devereux WL, Ocasio CA, Chen JK, Stearns T, Thomas RK, Dorsch M, Buonamici S, Watkins DN, Peacock CD and Sage J. A crucial requirement for Hedgehog signaling in small cell lung cancer. Nat Med 2011; 17: 1504-1508.