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Original Article

Long non-coding RNA HOST2 predicts poor prognosis and promotes cell proliferation and invasion in non-small cell lung cancer

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Abstract: Long noncoding RNAs (lncRNAs) have been considered recently as important players in tumor biology. In the present study, we aimed to elucidate the expression profiles and biological functions of lncRNA HOST2 in non-small cell lung cancer (NSCLC). Expression of HOST2 was evaluated by qRT-PCR in 78 NSCLC tissue samples and adjacent normal tissues. CCK-8 assay, flow cytometric analysis and transwell assay were performed to analyze the effects of HOST2 on NSCLC cell proliferation, apoptosis, migration and invasion in vitro. The effects of HOST2 on tumor growth in vivo were also evaluated. Our results suggested that the expression of HOST2 was significantly increased in the NSCLC tissues compared with the adjacent normal tissues. The increased HOST2 expression was also correlated to the aggressive clinicopathological features and poor prognosis of NSCLC patients. The experimental results revealed that the cell viability was enhanced by overexpression of HOST2 and the cell migration and invasion capacities were also promoted. The NSCLC cell apoptosis was enhanced significantly following the knockdown of HOST2. In vivo tumorigenesis assay showed that knockdown of HOST2 reduced the size and weight of the xenograft tumors. These findings indicated that lncRNA HOST2 plays an important role in regulating the pathogenic processes of NSCLC; therefore HOST2 might be a candidate target in the diagnosis and treatment of NSCLC.

Keywords: Non-small cell lung cancer, lncRNA, HOST2, prognosis, proliferation, invasion

Introduction

Lung cancer is one of the world leading causes of cancer-related mortality, and non-small cell lung cancer (NSCLC) accounts for 80-85% of the mortality of all lung cancers [1, 2]. Smoking is considered commonly to be the main cause of NSCLC. However, it is also observed in female never-smoke patients, particular in Asian countries [3]. Although rapid developments were made in chemotherapy, radiotherapy and surgical methods for NSCLC therapy in the past several decades, the 5-year survival rate was still no more than 15% [4, 5]. Thus, it is of critical importance to elucidate the underlying mechanisms involved in NSCLC at molecular levels which will benefit the development of effective diagnostic and therapeutic approaches of NSCLC.

Recently, long non-coding RNAs (lncRNAs) are considered to exert tumor suppressive and oncogenic functions in multiple human cancers [6, 7]. LncRNAs are the transcripts ranging from 200 nt to 100 kb but without the function as templates for protein synthesis [8, 9]. They mainly locate within nucleus or cytosolic compartment [10]. LncRNAs can be further subcategorized into the biotypes based on their location with respect to protein-coding genes [11]. LncRNAs regulate gene expression through epigenetic regulation, splicing, imprinting, transcriptional regulation and subcellular transport [12]. Apart from these, a couple of studies have proven that the deregulated lncRNAs might be closely correlated to the development and progression of cancers [9, 13]. Recent studies have verified the biological functions of lncRNA involved in the cancers such as renal cancer [14], ovarian cancer [15], gastric cancer [16], hepatocellular carcinoma [17], and lung cancer [18]. For example, MALAT1 can enhance cell migration of NSCLS cells by regulating the expression of motility-related genes [19, 20]. HOTAIR is correlated to the poor prognosis of human NSCLC, and the increased expression of

HOTAIR might be a cause of lung cancerous cell proliferation and migration [21]. Therefore, lncRNAs were considered to be the key regulators of NSCLC.

HOST2 is previously considered to be an important regulator of epithelial ovarian cancer [22] and hepatocellular carcinoma [23]. In the present study, we hypothesized that HOST2 might also exert oncogenic functions in NSCLC.

Materials and methods

Tissue specimens and patient characterization

78 pairs of histologically confirmed NSCLC tissues and adjacent non-tumor tissues were collected from patients who underwent the curative resections at the third Hospital of Hebei Medical University (Shijiazhuang, China). All patients did not receive any preoperative radiotherapy or chemotherapy. The collected samples were verified by two experienced pathologists. The specimens were rapidly frozen in liquid nitrogen and then transferred to the -80°C refrigerator for further analysis. After surgery, all patients were regularly followed up. This study was approved by the Ethics Committee of the third Hospital of Hebei Medical University. We obtained informed written consent from all participants enrolled in this study.

Cell culture and transfection

The normal human bronchial epithelial cell line (HBE) and four NSCLC cell lines, including A549, H1299, SPCA1 and H358, were purchased from Institute of Cell Biology of Chinese Academy of Science (Shanghai, China), and cultured in DMEM medium (GIBCO-BRL; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycinat 37°C in a humidified atmosphere with 5% CO_2.

To generate a HOST2 expression vector, we amplified a full-length HOST2 fragment by PCR. The PCR product was verified and subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen). To reduce the expression of HOST2, three HOST2 shRNA sequences (5'-GCGGCTCACCCTGCTACATTT-3', 5'-GCGCTCCCGGGTAGGCAATTG-3' and 5'-GCACCATTAAGCCATCTTTCT-3') and the negative control shRNA were subcloned into the pGIPZ vector (System Biosciences, Palo Alto, CA, USA). These plasmid vectors were transfected into NSCLC cells using Lipofectamine 2000 (Invitrogen). 48 hours post-transfection, transfection efficacy was analyzed by real-time quantitative RT-PCR (qRT-PCR).

RNA extraction and qRT-PCR

Total RNA was extracted from clinical tissue samples and cultured cells using Trizol Reagent (Invitrogen). The RNA was reverse transcribed into cDNA using the Primer Script RT reagent Kit (Takara, Dalian, China). qRT-PCR was performed using the Real-time PCR Mixture Reagent (Takara) on an ABI 7900 system (Applied Biosystems, Foster City, CA, USA). The sequences of the primers wereas followings: HOST2 forward, 5'-CTCAAATCAATCACGACCCT-3' and reverse, 5'-AATGTAGCAGGACGAGCC-3'; GAPDH forward, 5'-AGGTCGGAGTCAACGGATTTG-3' and reverse, 5'-TGTAAACCATGTAGTTGAGGTCA-3'. The qRT-PCR results were analyzed by the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH mRNA expression [24].

Cell proliferation assay

Cell proliferation was evaluated every 24 h for 4 days using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were allowed to grow in a 96-well plate and cultured at 37°C. Each well was added with 10 μl CCK-8 solution. Then, plates were incubated for another 2 h. The absorbance values of cells were measured using a microplate reader.

Transwell assay

For detection of cell migration and invasion, cells in 500 μL of serum-free medium were added to the upper chambers of an insert (8-μm pore size, BD Biosciences), which contained either uncoated or Matrigel-coated membranes. 500 μL medium with 10% FBS was added to the lower chamber as the chemoattractant. After 24 h of incubation, the cells remaining on the upper membrane were detached using cotton swabs. The cells located in the lower filters were fixed with methanol, and then stained with 0.1% crystal violet. Migrated or invaded cells were counted in five randomly by counting five random views under the microscope.

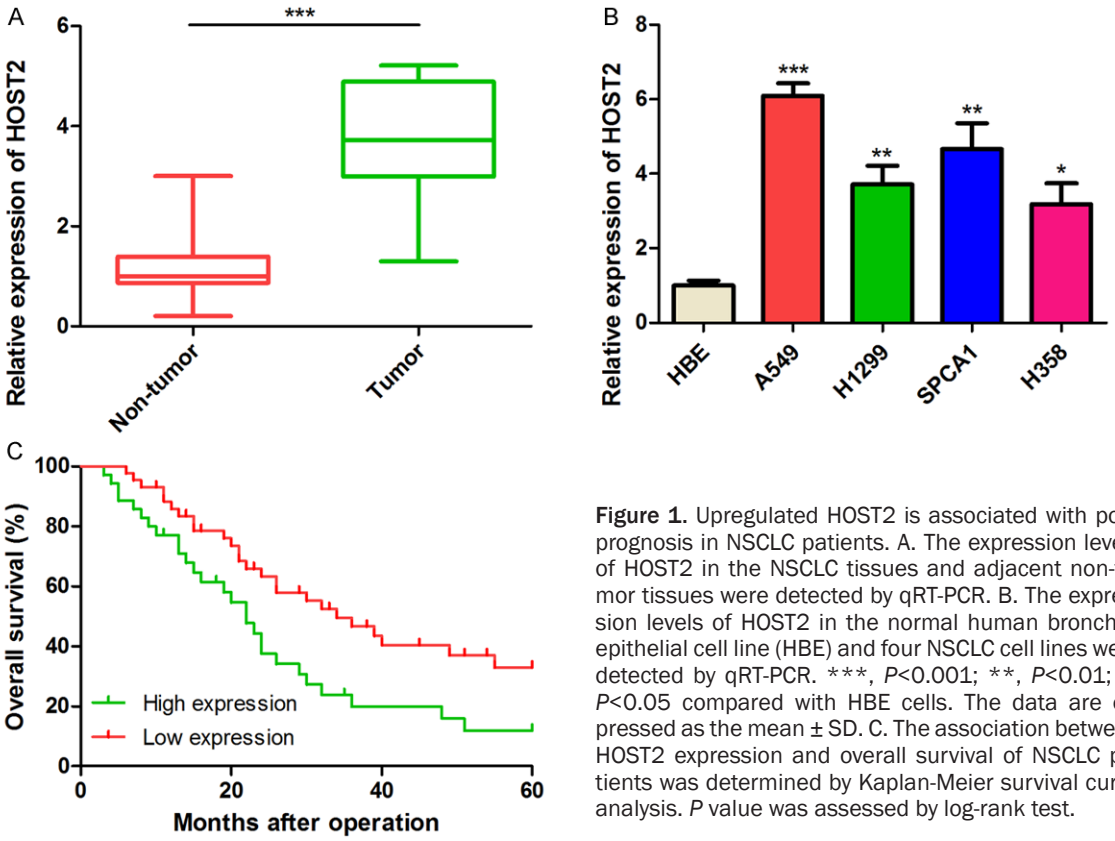

Figure 1. Upregulated HOST2 is associated with poor prognosis in NSCLC patients. A. The expression levels of HOST2 in the NSCLC tissues and adjacent non-tumor tissues were detected by qRT-PCR. B. The expression levels of HOST2 in the normal human bronchial epithelial cell line (HBE) and four NSCLC cell lines were detected by qRT-PCR. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ compared with HBE cells. The data are expressed as the mean ± SD. C. The association between HOST2 expression and overall survival of NSCLC patients was determined by Kaplan-Meier survival curve analysis. *P* value was assessed by log-rank test.

Flow-cytometric analysis of apoptosis

Cells were harvested 48 h after transfection by trypsinization. After double staining with FITC-Annexin V and propidium iodide, cells were analyzed by flow cytometry (FACScan; BD Biosciences) using Cell Quest software (BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells.

In vivo tumorigenesis assay

All experimental procedures were approved by the animal care and ethics committee of Hebei Medical University. Twelve five-week-old Balb/c-nu nude male mice, maintained in the specific pathogen-free (SPF) conditions, were randomized to the control or experimental group (6 mice/group). 3×10^6 NSCLC cells stably transfected with sh-HOST2 or sh-NC were implanted subcutaneously intothe back of each nude mice. Tumors were measured every five days and the volume was calculated following the formula: $V = 0.5 \times \text{length} \times \text{width}^2$. All mice were killed 4 weeks after injection, and the tumors were excised and weighed.

Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. The data are expressed as the mean ± SD. Two-tailed *t* test was used for comparisons of two independent groups. The chi-square test was used for the examination of correlation between HOST2 expression and the clinicopathological characteristics of NSCLC patients. Kaplan-Meier method and log-rank test were used for survival analysis. All statistical analyses were performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

HOST2 expression is increased in NSCLC

We first measured the expression levels of HOST2 in 78 pairs of NSCLC tissues and adjacent non-tumor tissues by qRT-PCR. The results showed that HOST2 expression was significantly increased in NSCLC tissues as compared

Table 1. Association of HOST2 expression with clinicopathological features of NSCLC patients

Characteristics	Total number (n=78)	HOST2 expression		*P* value
		Low (n=43)	High (n=35)	
Age (years)				0.733
≤60	34 (43.6%)	18	16	
>60	44 (56.4%)	25	19	
Gender				0.235
Male	48 (61.5%)	29	19	
Female	30 (38.5%)	14	16	
Tumor size (cm)				0.027
<3	33 (42.3%)	23	10	
≥3	45 (57.7%)	20	25	
TNM stage				0.005
I-II	36 (46.2%)	26	10	
III-IV	42 (53.8%)	17	25	
Tumor differentiation				0.747
Well	26 (33.3%)	15	11	
Moderate-Poor	52 (66.7%)	28	24	
Histology type				0.472
Adenocarcinoma	48 (61.5%)	28	20	
Squamous	30 (38.5%)	15	15	
Lymph node metastasis				0.003
Yes	46 (59.0%)	19	27	
No	32 (41.0%)	24	8	

with adjacent normal tissues (**Figure 1A**). Also, as shown in **Figure 1B**, the expression of HOST2 was markedly higher in the NSCLC cell lines (A549, H1299, SPCA1 and H358) than in the normal human bronchial epithelial cell line (HBE). A549 and H358 cells were selected for further studies.

Upregulated HOST2 is associated with poor prognosis in NSCLC patients

To further understand the significance of HOST2 in NSCLC, the correlation between HOST2 expression and the clinicopathological features of the 78 NSCLC patients was subsequently analyzed. According to the median ratio of relative HOST2 in NSCLC tissues, the 78 NSCLC patients were classified into two groups: high expression group (n=35; HOST2 expression ratio ≥4.0) and low expression group (n=43; HOST2 expression ratio ≤ 4.0). As recorded in **Table 1**, high expression of HOST2 in NSCLC tissues was significantly correlated with larger tumor size (*P*=0.027), advanced TNM stage (*P*=0.005) and lymph node metastasis (*P*=0.003). Furthermore, Kaplan-Meier analysis revealed that high HOST2 expression was significantly associated with shorter overall survival of NSCLC patients (*P*= 0.012; **Figure 1C**).

HOST2 regulates the proliferation of NSCLC cells

CCK-8 assay was performed to assess the role of HOST2 in regulating NSCLC cell proliferation. A549 cells were transiently transfected with three HOST2 shRNAs, which efficiently silenced endougenous expression of HOST2 (**Figure 2A**). sh-HOST2-1 was selected for further use in subsequent analysis. Also, ectopic expression of HOST2 in H358 cells by transfection with pcDNA3.1-HOST2 markedly increased the HOST2 level than that in pcDNA3.1-NC-transfected cells. As shown in **Figure 2B**, the NSCLC cells transfected with sh-HOST2 grew obviously shower compared to control cells, and the cell proliferation of pcDNA3.1-HOST2-transfected cells was significantly enhanced.

HOST2 regulates the apoptosis, migration and invasion of NSCLC cells

We then tested the effect of HOST2 on apoptosis through flow-cytometric analysis. As shown in **Figure 3**, the apoptotic rate of NSCLC cells transfected with sh-HOST2 was noticeably increased in comparison with control cells.

We investigated whether HOST2 exerts a functional role in facilitating NSCLC cell migration and invasion by transwell assay. As shown in **Figure 4**, NSCLC cells with ectopic expression of HOST2 enhanced the abilities of migration and invasion when compared to control cells, whereas reduced HOST2 suppressed cell migration and invasion. These data indicated that knockdown of HOST2 inhibits NSCLC cells migration and invasion.

Knockdown of HOST2 inhibits NSCLC tumorigenesis in vivo

To confirm the above data in vivo, A549 cells transfected with sh-HOST2 or sh-NC were

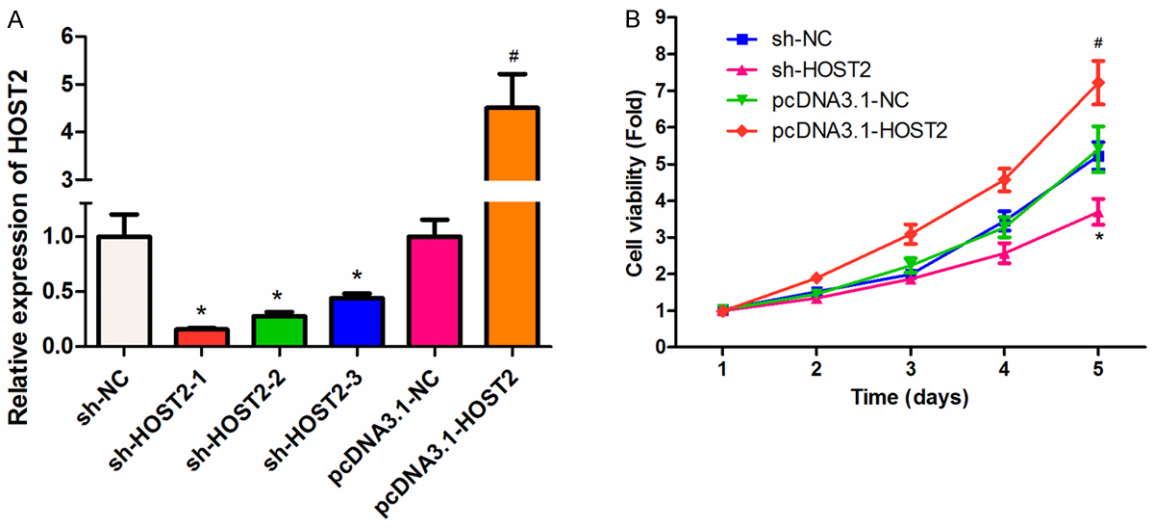

Figure 2. HOST2 regulates the proliferation of NSCLC cells. A. The transfection efficacy was verified by qRT-PCR. B. The viability of NSCLC cells after transfection was detected by CCK-8 assay. *, *P*<0.05 compared with sh-NC cells. #, *P*<0.05 compared with pcDNA3.1-NC cells. The data are expressed as the mean ± SD.

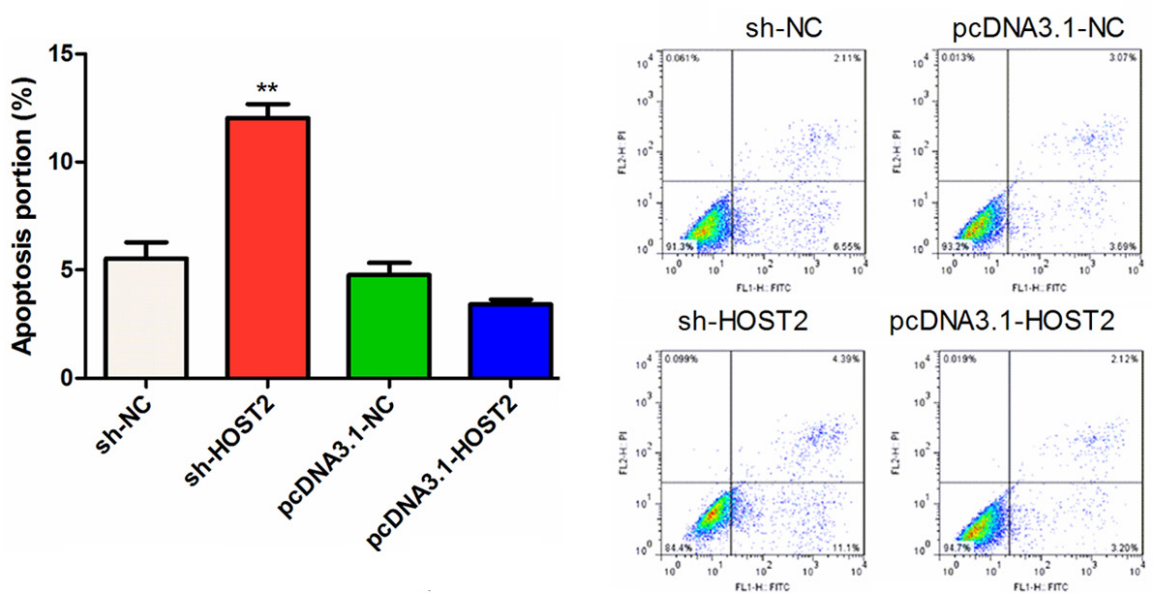

Figure 3. HOST2 regulates the apoptosis of NSCLC cells. The apoptotic rates of NSCLC cells after transfection were detected by flow-cytometric analysis. **, *P*<0.01 compared with sh-NC cells. The data are expressed as the mean ± SD.

injected subcutaneously into nude mice, respectively. Xenografts tumor volume was measured each week after palpable tumor formed, and mice were sacrificed 4 weeks after injection. As shown in **Figure 5A**, the tumors formed in sh-HOST2 group were substantially smaller than those in the sh-NC group. Moreover, the mean tumor weight at the end of the experiment was markedly lower in the sh-HOST2 group (**Figure 5B**). These results indicated silencing of HOST2 could inhibit tumorigenesis of NSCLC cells *in vivo*.

Discussion

In this study, we proved that the expression of HOST2 was increased in the tumor tissues of NSCLC patients. The increased levels of HOST2

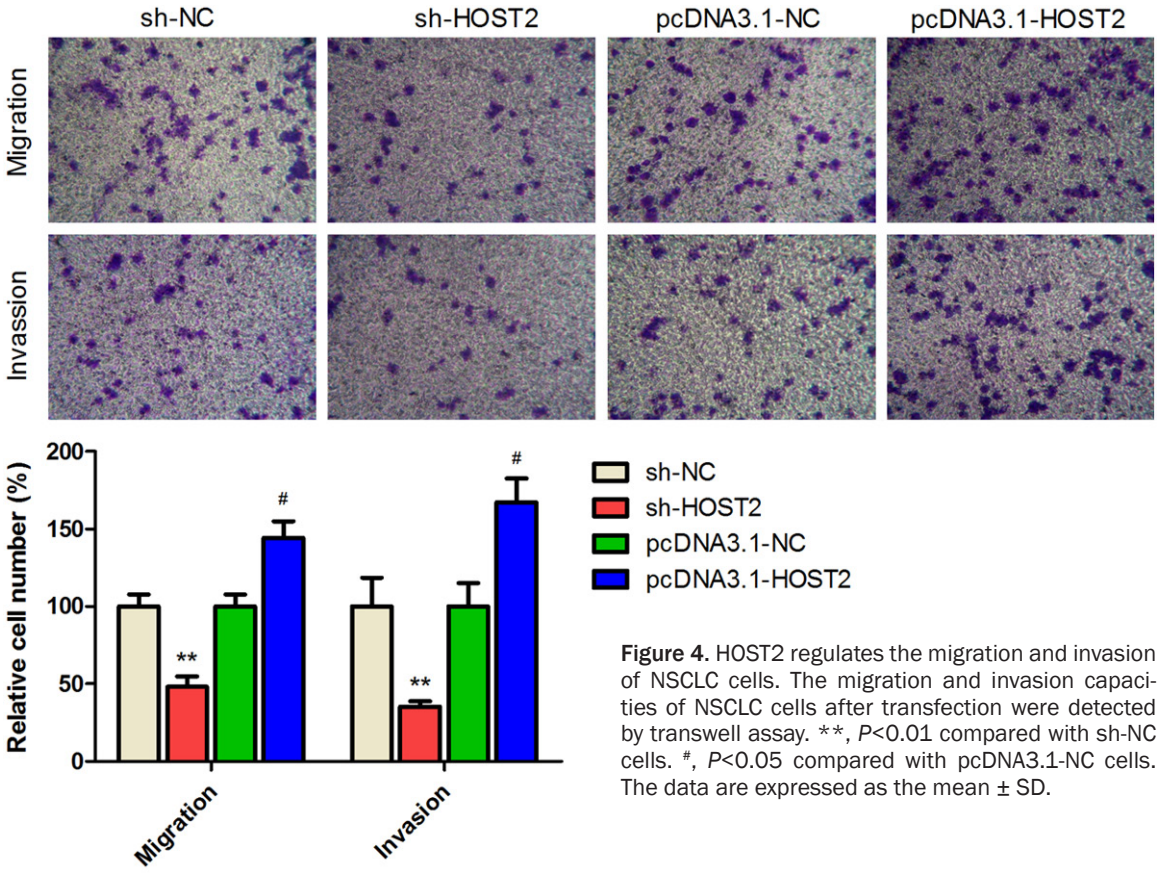

Figure 4. HOST2 regulates the migration and invasion of NSCLC cells. The migration and invasion capacities of NSCLC cells after transfection were detected by transwell assay. **, $P<0.01$ compared with sh-NC cells. #, $P<0.05$ compared with pcDNA3.1-NC cells. The data are expressed as the mean ± SD.

were correlated to the aggressive phenotypes and poor prognosis of NSCLC patients. HOST2 exerts its oncogenic functions through promoting the cancerous cell proliferation, migration and invasion. When it expression was silenced, the cell apoptosis was obviously increased, which has been also demonstrated in this study. The in vivo tumor formation study showed that the expression level of HOST2 was positively correlated to the tumorous size and weight.

HOST2 was identified to be specifically overexpressed in human ovarian cancer [25]. However, limited evidence has shown the relationship between HOST2 expression and NSCLC. Thus, we hypothesized HOST2 also involves the pathogenic process of NSCLC and it has been proven in our study. According to the current studies, the expression of HOST2 was correlated to the increased levels of claudin in the process of cancer development [26, 27]. As a member of HOST family, HOST2 is associated with the cancerous cell proliferation and differentiation. Its functions were performed according to the regulation of proteoglycan link protein (LP) that can specifically regulate the formation of extracellular matrix [28]. Under the regulation of LP, the intercellular coupling is enhanced and benefits for promoting cell differentiation. In addition, the HOST family can also regulate the function of metastasis molecule coded by SLC34A2. SLC34A2 was reported to be highly expressed in ovarian cancerous cells and thereby promoted the cell proliferation, migration and differentiation [29]. It is also known that the lncRNAs seek their targets and control the epigenetic trajectory contributing their ontogeny [30, 31]. The functions of some lncRNAs are considered to correlate to miRNAs [32]. It is previously reported that the level of miRNA let-7b was regulated by HOST2 [25]. When the activity of let-7b was inhibited, the mRNA and protein levels of HMGA2, c-Myc, Dicer and Imp3 were accordingly increased and thereby induces the cancerous cell metastasis and invasion. HMG2, c-Myc and Imp3 are the factors that related to the tumorous cells metastasis and infection. HMGA2 was involved in the regulation of multiple pro-metastatic fac-

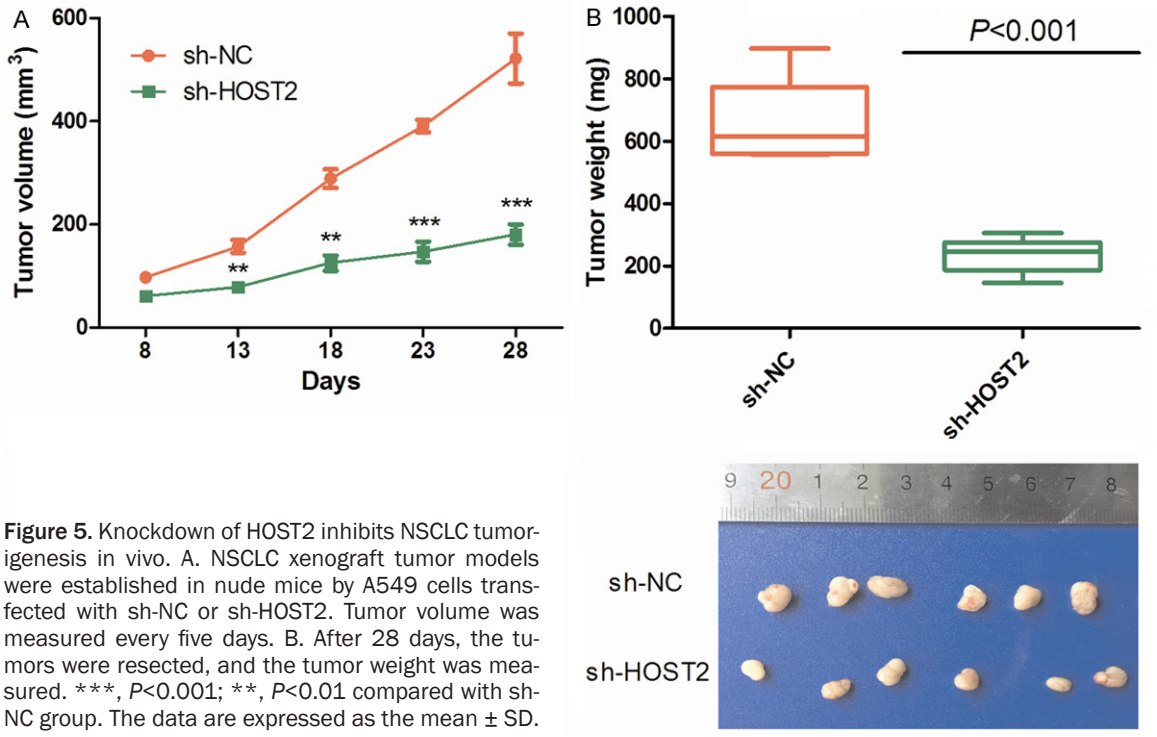

Figure 5. Knockdown of HOST2 inhibits NSCLC tumorigenesis in vivo. A. NSCLC xenograft tumor models were established in nude mice by A549 cells transfected with sh-NC or sh-HOST2. Tumor volume was measured every five days. B. After 28 days, the tumors were resected, and the tumor weight was measured. ***, $P<0.001$; **, $P<0.01$ compared with sh-NC group. The data are expressed as the mean ± SD.

tor genes and promotes the cell metastasis [33]. c-Myc can activate the process of formation and metastasis of tumors [34]. It can directly regulate the Lin28 gene and thereby inhibits the activity of let-7b. Consequently, the cancer progression was deteriorated [35]. Imp3, a key factor in the regulation of invasive cancerous cells, can target various genes related to the cell invasion and thereby enhances the capacity of movement and infection of the tumor cells [36]. Dicer is well-known in regulating the level of miRNA and thus related to the tumor cell activities [37]. On the other hand, the let-7 family can also target on genes related to cell cycling, such as CDK6 that normally induces cell cycle arrest in G1 phase [38]. Therefore, the role of HOST2 should be important. It directly or indirectly regulates the processes of cancer including cell proliferation, differentiation and metastasis and thereby promotes the deterioration of cancer.

In summary, we provided evidences that HOST2 can promote the processes of NSCLC cells and its increased expression led to poor prognosis of NSCLC patients. Therefore, HOST2 can be a candidate biomarker used for diagnosis and treatment of NSCLC. Although the increased expression of HOST2 was found in NSCLC patients, the number of the paired specimens is still limited. In addition, the underlying mechanisms of HOST2 were not understood clearly. It requires further validation of the downstream targets of HOST2 to present more comprehensive molecular mechanism of HOST2.

Disclosure of conflict of interest

None.

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