

# Nm23-H1 was involved in regulation of KAI1 expression in high-metastatic lung cancer cells L9981

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**Background:** The tetraspanin KAI1/CD82 was identified as a tumor metastasis suppressor that down-regulated in malignant progression of lung cancer. However, the underlying mechanism of anti-metastasis role of KAI1 in lung cancer is hardly known. In this paper, we sought to study the function and regulatory mechanism of KAI1 in high metastasis lung cancer cell line.

**Methods:** KAI1 expression was detected in high/low metastatic large lung cancer cell line L9981/NL9980 by quantitative real-time polymerase chain reaction (qRT-PCR). The tumor suppressor function of KAI1 was determined by wound healing assay after over-expression or knockdown of KAI1 in L9981 or NL9980 cells. Invasion assay was performed to detect the invasion ability of L9981 by transfection of KAI1. The effect of tumor suppressor p53 on KAI1 expression was measured by western blot and luciferase assay. Then the regulation of KAI1 due to over-expression of metastasis suppressor nm23-H1 was monitored by qRT-PCR, western blot and reporter gene assay. The progression of L9981 cells after p53 and nm23-H1 expression was detected by invasion assay. Also, methylation status of KAI1 promoter in NL9980 and L9981 cells were examined by bisulfite sequencing and methylation-specific PCR.

**Results:** We found that KAI1 is down-regulated in high metastatic L9981 cells compare with NL9980 cells. The migration and invasion of L9981 cells were remarkably suppressed *in vitro* by KAI1 transfection. The migration ability of NL9980 was enhanced by inhibition of KAI1. Furthermore, KAI1 expression was induced after over-expression of p53 or nm23-H1, while cell invasion was inhibited in L9981 cells. The results of reporter analysis indicated that KAI1 promoter region between -922 to -846 could response to nm23-H1. In addition, we discovered only slight methylation of KAI1 promoter, which showed that loss expression of KAI1 in L9981 cells may not due to promoter methylation.

**Conclusions:** The results suggested that nm23-H1 was involved in the KAI1-regulated inhibition of metastasis in lung cancer cells. More insights into the relationship between KAI1 and other metastasis suppressors will pave the way for the elucidation of anti-metastasis mechanism in lung cancer.

**Keywords:** KAI1; lung cancer; nm23-H1; L9981

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## Introduction

Lung cancer is one of the most prevalent malignancies worldwide, with more than 1 million deaths every year. Invasion and metastasis of tumor cancer is a complex, multi-step process that numerous cellular genes were involved. Many lung cancer patients develop metastatic diseases, which are the main cause of death (1,2). However, there is less effective method for early detection, and the highly metastatic ability might lead to the mortality of lung cancer.

The metastatic suppressor Nm23-H1 is highly conserved in eukaryotic species, which possess various biological functions, such as transcriptional regulation, proliferation, differentiation, and tumor metastasis suppression. Down-regulation of Nm23-H1 expression level was observed at late stages with high metastatic tumors. It was reported that Nm23-H1 interacts with a number of cellular proteins involved in signaling pathways and oncoproteins of tumor viruses (3,4). Therefore, the elucidation of these interrelationships could be useful to study the metastasis suppressor gene ability of Nm23-H1, as well as the function of the partner protein themselves.

The function of KAI1 (CD82) in cancer progression was analyzed using genetic screening in a prostate cancer cell line aim to identify metastasis suppress genes (5). Down-regulation of KAI1 expression was associated with advanced stages of many malignancies including prostate, colon, lung, pancreatic, breast, ovarian and other cancers (6,7). KAI1 belongs to transmembrane 4 superfamily (TM4SF), encoding a 267 amino acid glycosylated membrane protein with four highly conserved transmembrane domain. Previous study revealed the existence of CpG island in the 5'-promoter of KAI1, which extend to the first exon and first intron (8), indicating the possible methylation of KAI1 promoter. It was noted that KAI1 play an important role in cell proliferation, adhesion and movement. KAI1 suppress metastasis through its inhibition of cell movement and adhesion (9,10). Recently, it was reported that no co-localization was observed between a KAI1 splice variant and E-cadherin. Moreover, this KAI1 variant promotes tumor progression and cancer metastasis (11). Besides, KAI1 could regulate MMP-2 and MMP-9 level in patients with endometrial cancer. The tumor development might be influenced by crosstalk between these proteins (12). HBV has been found to inhibit KAI1 by hypermethylation of KAI1 promoter, which maybe a key step in hepatocellular carcinoma progression (13). In addition, there exists

a correlation between KAI1 and VEGF expression in the serum or tissue sample in HCC patients (14). The inhibition of KAI1 was related to EGFR over-expression, as well as EGFR mutations in non-small cell lung cancers (15). KAI1 suppress metastasis by inhibition of Wnt signaling pathway through up-regulation of miR-203 and directly down-regulation of FZD2 in lung cancer (16). Thus, it was accepted that KAI1 suppresses tumor metastasis via multiple signaling pathway and considered as a valuable marker for assessing metastasis potential (17). Nonetheless, the regulatory mechanism of KAI1 and its interrelationship with other factors in lung cancer is not fully elucidated.

In this study, we discovered different expression level of KAI1 among diverse lung cancer cells. The results showed a reduction of KAI1 in high metastasis potential L9981 cells. Further we demonstrated inhibition of migration and invasion ability of L9981 by KAI1 transfection. The migration of NL9980, a cell line with low metastatic potential, was enhanced by inhibition of KAI1. Next, p53 was found to activate KAI1 promoter and enhance KAI1 expression. Up-regulation of KAI1 was also observed by over-expression of nm23-H1 in L9981 cells. Moreover, over-expression of p53 and nm23-H1 lead to inhibition of invasion. The motif between -922 and -846 was identified as responsive sites for nm23-H1 in KAI1 promoter. Nevertheless, no obvious methylation of KAI1 promoter was detected by bisulfite sequencing and methylation specific PCR in L9981 and NL9980 cells. The findings of the present study implied a synergistic mechanism of tumor suppressors in lung cancer metastasis.

## Methods

### *Cell culture and transfection*

NL9980 and L9981 were isolated and established from a human lung large cell carcinoma cell line (WCQH-9801). The L9981 cells have low expression of nm23-H1 and higher invasion and metastasis potential (18). L9981-nm23-H1 cell line was established by infected virus with *nm23-H1* gene (19). Other lung cancer cell lines include A549, GLC-82, A2, SPCA-1, NCI-H460, SH-77, NCI-H446, LTEP- $\alpha$ -2, YTMLC-9 and normal lung cell line MRC-5. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO, USA) at 37 °C with 5% CO<sub>2</sub> incubator. Cell transfection was carried out using Lipo2000 (Invitrogen, CA, USA) according to instruction manual.

**Table 1** Primer sequences for plasmids construction

Plasmid	Forward (5'-3')	Reverse (5'-3')
pCMV-KAI1	CGGAATTCATGGGCTCAGCCTGTATC	CCCAAGCTTCTCAGTACTTGGGGACCTTG
pGL3-922	CTAGCTAGCGTTCTGGGCTACTTCCCCAGGGGCC	CCCAAGCTTTGGAATTCACCTGGTTCAC
pGL3-730	TGCTAGCGAATTC AAGGGCTAGA ACTG	CCCAAGCTTTGGAATTCACCTGGTTCAC
pGL3-600	TGCTAGCTAGGACACAAACCGCTCCC	CCCAAGCTTTGGAATTCACCTGGTTCAC

### Plasmids and siRNA

KAI1 expression plasmid, pCMV-KAI1, were constructed by PCR of full-length KAI1 gene and cloned into pCMV-Tag2B vector. For the construction of pGL3-922, KAI1 promoter sequences from -922 to +353 were amplified from human genomic DNA and cloned into reporter plasmid pGL3-basic. Plasmids pGL3-730 and pGL3-600 were constructed by amplifying promoter sequences from pGL3-922, then cloned the products into pGL3-basic. The primers were listed in *Table 1*. Plasmid pGL3-922-p53m was constructed as follows: PCR was conducted for the first round with forward primer 5'-TACTTCCCCAGGGGCCAGTCCAGGAGACTTCAGC--CTGT-3' and reverse primer 5'-CCCAAGCTTTGGAATTCA CCTGGTTCAC-3' using pGL3-922 as template. Then the PCR product was used as template for the second round of PCR with the forward primer 5'-CTAGCTAGCGTTCTGGGCTACTTCCCCAGGGGCC-3' and reverse primer 5'-CCCAAGCTTTGGAATTCACCTGGTTCAC-3'. The PCR product from the second round was cloned into pGL3-basic. Plasmid pGL3-846 was constructed as follows: DNA fragment of promoter sequence from -846 to +353 was generated by digestion of pGL3-922 with *Bam*H I and *Hind* III, then the fragment was ligated with *Bgl* II and *Hind* III double-digested pGL3-basic vector. All the constructions were verified by sequencing. pEGFP-p53 was purchased from Clontech. KAI1 siRNA was purchased from Ribobio (Guangzhou, China).

### Cell Migration and invasion assays

Cell migration assay was carried out using wound-healing assay. Briefly, cells were cultured in twelve-well plates. Twenty-four hours post transfection, a plastic micropipette tip was used to generate a wound area across the center of the well, and the floating debris were washed with PBS and cultured in serum-free medium. Width of the wound was

measured at indicated time points. Three to four wound areas were investigated and photographed under a phase-contrast inverted microscope at 40× magnifications.

The cell invasion ability was determined using BD matrigel invasion chambers according to manufacture's protocol. Briefly, sixteen hours post transfection, cells were seeded onto upper chambers. After incubation for 48 h at 37 °C, stain of invasion cells was carried out using 0.1% crystal violet after fixation, and five random fields from each insert were counted at 200× magnifications. The invasion assay was conducted in duplicate in two separate experiments.

### Quantitative real-time polymerase chain reaction (PCR)

For real-time PCR, 1.0 µg of total RNA was used with hexamer random primers for the first strand cDNA synthesis by MLV Reverse Transcriptase (Takara, Japan). The PCR reaction was carried out using the ABI Prism 7900HT fast RT-PCR system (Applied Biosystems, Life technologies, USA). In each experiment, all the samples were tested in triplicates. The relative amount of mRNA was calculated by the  $2^{-\Delta\Delta Ct}$  method after normalization to endogenous GAPDH mRNA levels. Primer sequences to detect KAI1 and GAPDH expression were designed refer to published paper (20).

### Antibodies and Western blot

All the antibodies were purchased from Santa Cruz. The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA; 1 mM PMSF; 1 mg/mL aprotinin). Protein concentrations were measured using BCA method. The cell lysates were electrophoresed on 10–15% SDS-PAGE gel, transferred to nitrocellulose membrane. The membranes were blocked with 5% skimmed milk in PBS-Tween (0.1%) for one hour. Then the membranes were probed with antibodies against KAI1, p53 and nm23-H1 at dilution of 1:1,000, and  $\beta$ -actin at dilution of 1:2,000,

**Table 2** Expression level of KAI1 in normal cell and different human lung cancer cell lines

Cell lines	$\Delta\text{Ct} (\bar{x}\pm s)$
MRC-5	5.10 $\pm$ 0.10
L9981	15.30 $\pm$ 0.46
NL9980	7.53 $\pm$ 0.68
A549	11.83 $\pm$ 0.41
GLC-82	12.1 $\pm$ 0.44
A2	12.77 $\pm$ 0.42
SPCA-1	10.63 $\pm$ 1.63
NCI-H460	15.63 $\pm$ 0.42
SH-77	22.77 $\pm$ 1.37
NCI-H446	9.60 $\pm$ 1.23
LTEP- $\alpha$ -2	13.17 $\pm$ 0.50
YTMLC-9	11.2 $\pm$ 1.25

followed by incubation at 4 °C overnight. After incubation with HRP-conjugated secondary antibody for 1 h, the blots were visualized with Chemiluminescent HRP Substrate (Millipore).

### Luciferase assay

L9981 cells were plated in 24-well plate at  $1\times 10^5$  cells/well. The cells were co-transfected with 200 ng of pGL3-922, pGL3-922-p53m or pGL3-Basic, and 100 ng of pEGFP-p53 or pEGFP vector. Co-transfection of L9981 or L9981-nm23-H1 cells was conducted using 200 ng of reporter plasmids with different length of KAI1 promoter. Forty-eight hours after transfection, cells were lysed and assayed for luciferase activity using Dual-luciferase glow assay (Promega, Madison, WI) according to manufacturer's protocol. All transfection experiments were carried out in triplicate.

### Bisulfite sequencing

Genomic DNA was extracted from NL9980 and L9981 cell lines using the Genome DNA extraction kit (Takara) according to manufacturer's instructions. Bisulfite conversion of 1 $\mu$ g DNA was conducted with Methylamp DNA Modification Kit (Epigentek). The DNA samples were used for the PCR amplification by hot-start Taq DNA polymerase (Takara). Two domains of *KAI1* gene were subjected to sequencing. The primers for the first domain (-474 to -190) were 5'-GTAGGGTAGGGTAGGATTAGGAA-3' as sense primer and 5'-ACCAACCTCACCCCAAACCAAC-3'

as antisense primer. The primers for the second domain (-156 to +126) and PCR condition have been described previously (21). The PCR products were cloned into pMD18T vector (Takara) and subjected to sequencing. Five clones of each domain were selected for sequence analysis.

### Methylation-specific polymerase chain reaction

The primers and conditions for the MSP analysis of KAI1 promoter have been described (22). The un-methylated primers were 5'-ATAGAGGAGAGATTTTGTAGT-3' (forward) and 5'-CCCAAACTCAATCACTCCTA-3' (reverse), the methylated primers were 5'-ATAGAGGAGAGATTTTCGTAGC-3' (forward) and 5'-CCGA AACTCAATCACTCCTC-3' (reverse). Bisulfite modified genomic DNA was amplified by MSP at least twice using hot-start Taq DNA polymerase (Takara) and the PCR products were subjected to agarose gel analysis. Controls of non-methylated template and methylated template were purchased from Qiagen.

### Statistical analysis

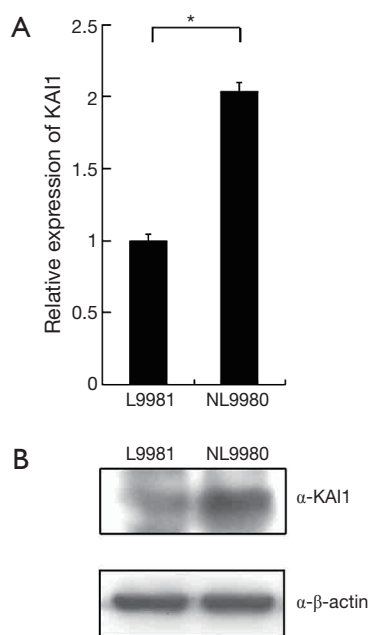
The data were presented as mean  $\pm$  standard deviation (SD). The statistical comparisons of control and treatment groups were analysis by Student's *t*-test. Data processing was carried out using SPSS15.0 software. The differences with  $P<0.05$  were considered statistically significant.

## Results

### *KAI1* is down-regulated in high metastatic lung cancer cells L9981

To study the regulatory mechanism of KAI1 in human lung cancer tumorigenesis, KAI1 expression level was detected by real time PCR in different lung cancer cell lines. The data showed in *Table 2* indicated that expression level of KAI1 mRNA in different types of lung cancer cell lines is remarkably lower than those in normal human fetal lung fibroblast cell line MRC-5. Moreover, the expression of KAI1 mRNA in the human large cell lung cancer cell lines with opposite metastatic potential is also different. As sub-cell lines with high or low metastatic and invasion ability, we further analyzed the KAI1 expression level in L9981 and NL9980 by real time PCR and western blot assay. Results showed that both mRNA and protein level were lower in L9981 than NL9980 (*Figure 1*). L9981 is a



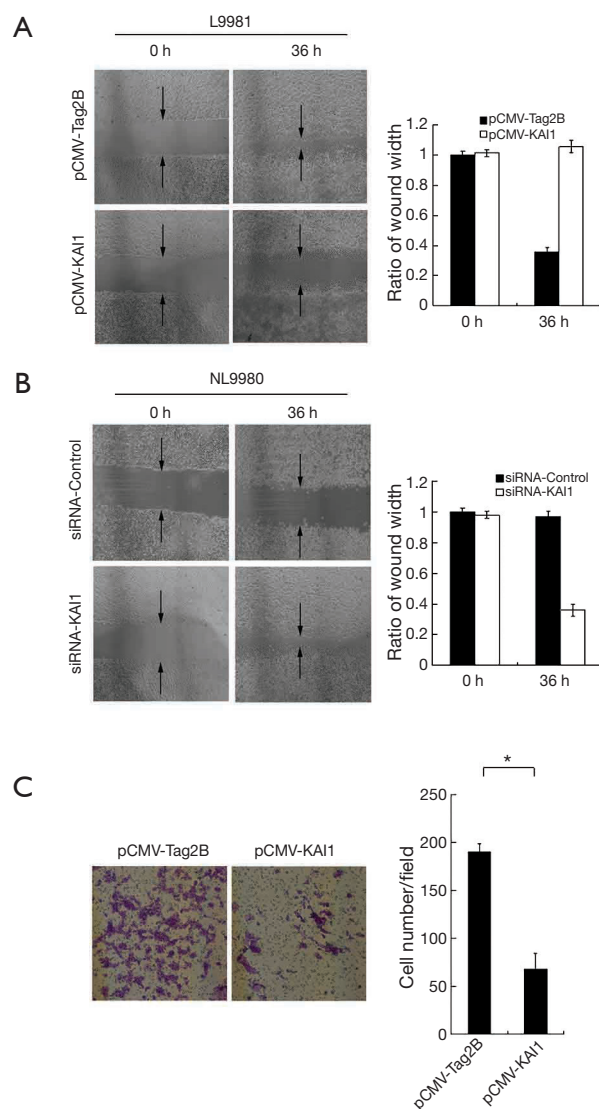


**Figure 1** KAI1 expression is down-regulated in L9981 compared with NL9980 cells. (A) Relative expression of KAI1 in high metastasis potential L9981 and NL9980 cells were measured by quantitative real time PCR; (B) protein level of KAI1 in L9981 and NL9980 were detected by western blot. (\* $P < 0.01$ , Student's  $t$ -test).

high metastasis cell line compared with NL9980. Thus, the results here suggested that down-regulation of KAI1 expression maybe related with high metastasis phenotype in human lung cancer cells.

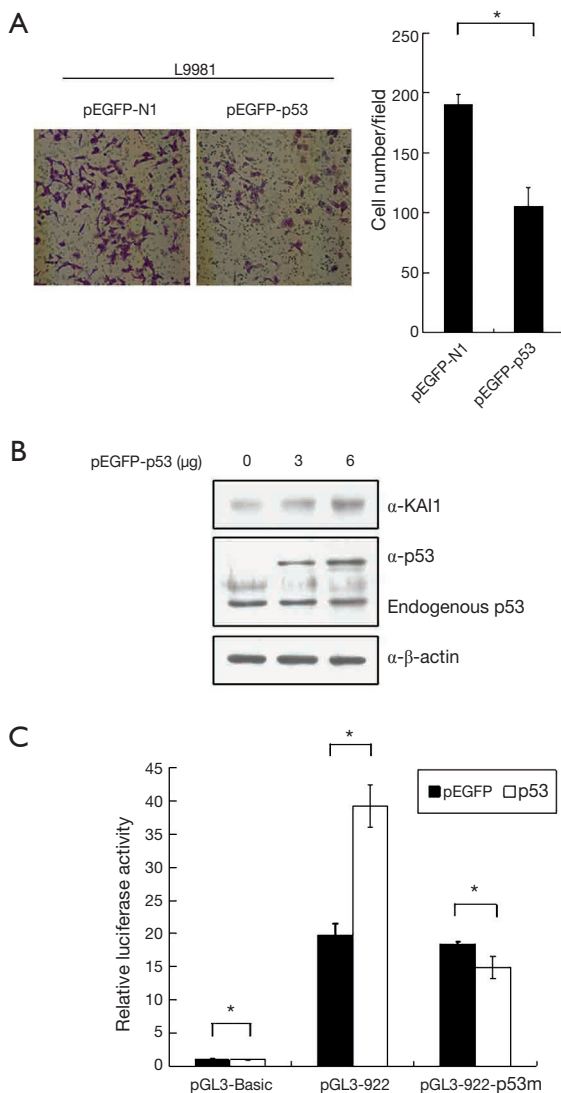
### *KAI1 suppresses migration and invasion of L9981*

To evaluate whether KAI1 suppress metastasis of lung cancer cells L9981, we examined the effect of KAI1 on migration of lung cancer cells by wound healing assay. The L9981 cells were transfected with pCMV-KAI1 or the pCMV-Tag2B vector, and cell migration was detected after 36 h. As shown in *Figure 2A*, the cell motility was decreased after KAI1 was over-expressed. Moreover, the migration of NL9980 cells was enhanced by inhibition of KAI1 (*Figure 2B*). To determine another important step in cancer cell metastasis, we analyzed whether KAI1 has influence on cell invasion of L9981, which was assessed by invasion assay. Cells were transfected with pCMV-KAI1 and the vector plasmid, the invasive cells were stained and counted under microscope. In compare with the control group, a significant decrease of invasive cells in KAI1



**Figure 2** KAI1 suppresses migration and invasion ability of L9981 cells. (A) Wound healing assay was performed to study migration. L9981 cells were transfected with pCMV-KAI1 or pCMV-Tag2B vector, cell migration was determined to study migration ability; (B) NL9980 cells were transfected with siRNA-KAI1 or siRNA-control, cell migration was determined to study migration ability; (C) L9981 cells were transfected with pCMV-KAI1 or pCMV-Tag2B vector and invasion assay was carried out to study invasion ability. The data shown as the means  $\pm$  SD. (\* $P < 0.01$ , Student's  $t$ -test).

over-expressed group was observed (*Figure 2C*). These results were in accordance with the wound healing assay. Collectively, our data implied that over-expression of KAI1 could inhibit progression of high metastasis L9981 cells.



**Figure 3** Up-regulation of *KAI1* expression by p53 in L9981 cells. (A) L9981 cells were transfected with pEGFP-N1 or pEGFP-p53, cell invasion was determined to study invasion ability; (B) pEGFP-p53 was transfected into L9981 cells. Protein level of *KAI1* and p53 were examined by western blot; (C) L9981 cells were co-transfected with pGL3-Basic, pGL3-922 or pGL3-922-p53m, and pEGFP-p53 or pEGFP vector. Reporter gene assay were carried out forty-eight hours post transfection. The data shown as the means  $\pm$  SD. (\* $P < 0.05$ , Student's *t*-test).

### *p53* increase *KAI1* expression level

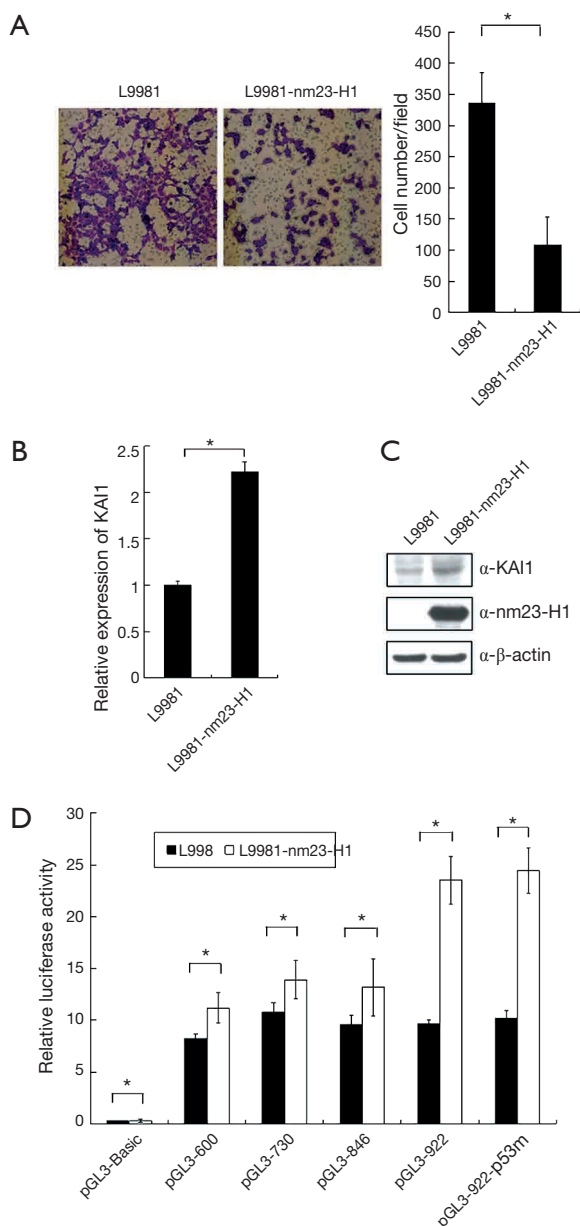
Recently, it was noted that *KAI1* expression can be regulated by p53 (23). We sought to examine this issue in high-metastatic lung cancer cell lines. The results in *Figure 3A*

suggested that invasion ability of L9981 cells was inhibited by over-expression of p53. Then increasing amounts of pEGFP-p53 expression plasmid were transfected into L9981 cells, and western blot assay was carried out to assess the *KAI1* expression level. Results showed in *Figure 3B* demonstrated that over-expression of p53 can up-regulate *KAI1* protein level in L9981. To understand whether *KAI1* promoter activity was involved in this up-regulation effect, reporter plasmid containing *KAI1* promoter region from -922 to +353 was constructed, which the published p53 binding site was included. Results of luciferase activity assay revealed that *KAI1* promoter can be activated by p53. However, mutation of the p53 binding site in *KAI1* promoter abolished this activation effect (*Figure 3C*), indicating the induction of *KAI1* expression by p53 in L9981 cells.

### *Nm23-H1* increase *KAI1* expression in L9981 cells

In order to confirm a cross-talk between *KAI1* and nm23-H1, L9981-nm23-H1 was established by stable transfection of nm23-H1 into L9981 cells. We discovered that the invasion ability of L9981-nm23-H1 was inhibited compare with L9981 cells (*Figure 4A*). The mRNA and protein level of *KAI1* was measured by real time PCR and western blot in L9981 and L9981-nm23-H1 cells. We found that *KAI1* expression, at both mRNA and protein level, is higher in L9981-nm23-H1 than those of L9981 cells (*Figure 4B and C*). Hence, these results indicated that expression of nm23-H1 lead to up-regulation of *KAI1* in L9981-nm23H1 cells.

Next, to study whether nm23-H1 alter *KAI1* promoter activity, reporter plasmid pGL3-922, which carrying *KAI1* promoter, was transfected into L9981 and L9981-nm23-H1 cells. Forty-eight hours post transfection, cells were lysed and luciferase activity was assayed. As shown in *Figure 4D*, expression of nm23-H1 result in an increase of luciferase activity in L9981-nm23-H1 compared with L9981 cells, which indicated that *KAI1* reporter activity can be induced by expression of nm23-H1. To find the promoter domain responsible for nm23-H1 activation, we constructed a series of reporter plasmids with different length of *KAI1* promoter. L9981 and L9981-nm23-H1 cells were transfected with these reporter plasmids. The results of luciferase assay demonstrated that only pGL3-922 is able to induced by nm23-H1 expression (*Figure 4D*), while activation of other truncated promoters (pGL3-846,730 and 600) was weakly detectable. Therefore, it was probable that the responsive element for nm23-H1 in *KAI1* promoter was



**Figure 4** Up-regulated of KAI1 expression by nm23-H1. (A) Invasion assay of L9981 and L9981-nm23-H1 cells were carried out to study invasion ability; (B) the mRNA level of KAI1 was detected by quantitative real-time PCR in L9981 or L9981-nm23-H1 cells; (C) the protein level of KAI1 was detected by western blot in L9981 or L9981-nm23-H1 cells; (D) luciferase assay was conducted to identify the domain responsible for nm23-H1-mediated activation upon KAI1 promoter. Reporter plasmids harboring KAI1 promoter or truncations were transfected into L9981 or L9981-nm23-H1 cells. Reporter gene assay were carried out forty-eight hours post transfection. The data shown as the means  $\pm$  SD. (\* $P < 0.05$ , Student's *t*-test).

between -922 to -846. Interestingly, p53 binding site is dispensable for nm23-H1-mediated stimulation of KAI1 promoter (Figure 4D), which showed that KAI1 can be activated by nm23-H1 without intact p53 binding site.

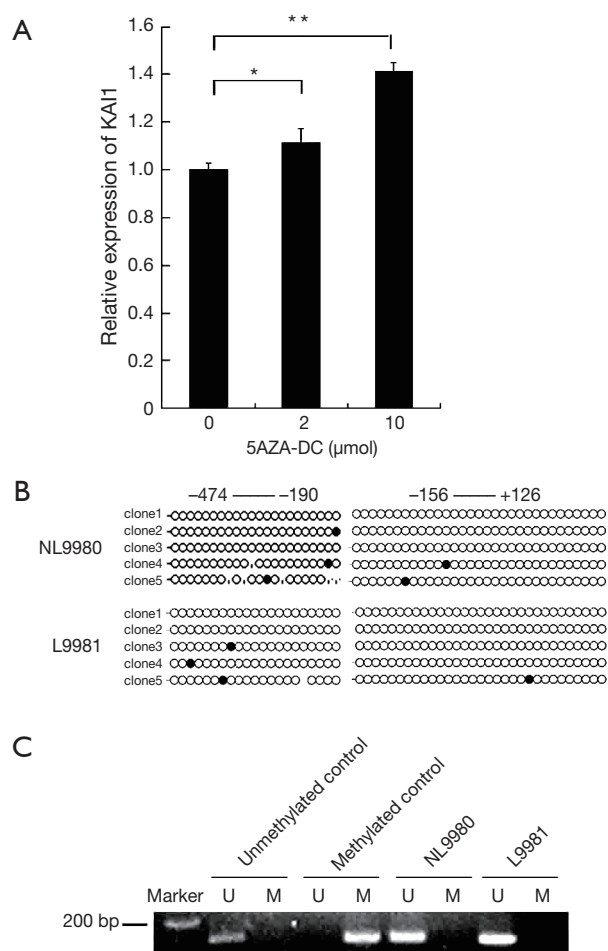
#### Methylation is not significant for KAI1 expression in L9981 cells

It was unclear whether KAI1 promoter activity was regulated by methylation in L9981 cells, thus we applied 5-AZA-DC, a methyltransferase inhibitor, to analyze KAI1 expression. We observed restoration of KAI1 level by 5-AZA-DC treatment in L9981 cells (Figure 5A). This result indicated that promoter methylation might be involved in the down-regulation of KAI1 in lung cancer cells. Next, the methylation status of KAI1 promoter was studied using bisulfite sequencing and methylation specific PCR (MSP). Two domains, -474 to -190 and -156 to 126 of KAI1 promoter, were subjected to bisulfite sequencing. However, results revealed that CpG island in KAI1 promoter does not exhibit high methylation level in L9981 or NL9980 cells (Figure 5B). Correlate with results of bisulfite sequencing, no methylation was observed in MSP analysis (Figure 5C). Taken together, it thus implicates that KAI1 expression maybe mediated through other pathway, rather than methylation of promoter in these two cell lines.

#### Discussion

Although many breakthroughs were achieved for early detection and personalized therapy of lung cancer, it is still the leading cause of death in the world. Notably, primary cancer may not be the reason for the death, whereas a majority of patients die of tumor metastasis. As a complicated and multi-level biological process, further research is required to study the molecular mechanism of metastasis in lung cancer. It was known that metastasis suppressors exert their functions by inhibiting motility and invasiveness of tumor cells. Aside from manipulating the cellular affinity of tumor alone, co-operation of different suppressors may occur to inhibit cancer metastasis.

It was recently reported that KAI1 and KISS1 (a metastasis suppressor gene) expression was significantly decreased in breast cancer in comparison with normal tissue, especially in patients with aggressive tumors (24). Moreover, Wu *et al.* observed a positive relation between KAI1 and E-cadherin expression in NSCLC, and down-regulation of these two proteins was closely related to differentiation, pTNM stages



**Figure 5** Analysis of *KAI1* promoter methylation. (A) L9981 cells were treated with 5-AZA-DC and mRNA level of *KAI1* was detected by quantitative real-time PCR; (B) bisulfite sequencing of two domains of *KAI1* promoter in NL9980 and L9981 cells. Hollow circle—non-methylated CpG; solid circle—methylated CpG; dot—uncertain; (C) methylation of *KAI1* promoter in NL9980 and L9981 cells were analyzed by methylation-specific PCR. The data shown as the means  $\pm$  SD. (\* $P < 0.05$ , \*\* $P < 0.05$ , Student's *t*-test).

and metastasis (25). The study on the expression of HIF-1 $\alpha$  and VEGF demonstrated that they were negatively regulated by *KAI1* in the human prostate cancer cell line PC3, which contribute to inhibition of cancer metastasis (26). Besides, *KAI1* was activated by recruiting histone acetyltransferase Tip60 in the presence of PMA (27). Therefore, *KAI1* exerts profound metastasis-suppressor activity in the tumor malignancy process.

Previous reports have proved that *KAI1* expression is

down-regulated in many cancers. Hence, we compared *KAI1* expression in various lung cancer cells, and a pair of high and low metastatic potential cell lines (L9981 and NL9980). The results showed decreased mRNA and protein level of *KAI1* in L9981 cells compared to NL9980 cells. These data correlate well with the founding that expression of *KAI1* was down-regulated in several cancers with advanced stage, including ovarian, colon and lung (7). There was evidence revealed that *KAI1* and nm23-H1 were down-regulated simultaneously in tumor progression of non-small cell lung cancer (28). Besides, *KAI1* expression was decreased in basal cell carcinomas, while nm23-H1 was up-regulated (29). It was reported that expression of *KAI1* and nm23-H1 were correlated with prognoses of pancreatic cancer and papilla of vater cancer (30). Thus we detected *KAI1* level in L9981 and L9981-nm23-H1, a stable nm23-H1 expression cell line. The results implied that *KAI1* expression can be up-regulated by nm23-H1. Because low level of nm23-H1 is frequently observed in the progression of several cancers (31,32), our results indicated that loss expression of nm23-H1 might contribute to the down-regulation of *KAI1*.

To identify the responsive motif for nm23-H1 in *KAI1* promoter, a series of plasmids with different length of *KAI1* promoter were constructed and reporter assay were carried out. Interestingly, we found that nm23-H1 may bind to the *KAI1* promoter between -922 to -846, a domain that probably carrying AP1, AP2 and p53 binding sites (33). It has been reported that nm23-H1 was positively regulated by p53 via removing of Mdm2, a negative regulator of p53, from p53-Mdm2 complex (34), and *KAI1* expression can be up-regulated by p53 (35). Results of invasion assay showed an inhibition of L9981 invasive ability by over-expression of p53. We further demonstrated that p53 can up-regulated *KAI1* expression through promoter activation in L9981 cells. These data indicate the possibility that p53 promote the expression of *KAI1*, then lead to the inhibition of invasion in L9981 cells. By contrast, we found p53 binding site is not indispensable for *KAI1* promoter induction by nm23-H1, which indicated that nm23-H1 activate *KAI1* promoter in a p53-independent manner in L9981 cells.

The methylated modification of promoter contributes to loss of expression of several tumor suppressors. High methylation status of *KAI1* was observed in myeloma cell lines and the methyltransferase inhibitor 5'-aza-CdR is able to promote *KAI1* expression in U266 cells (22). There are also reports suggested *KAI1* gene was unmethylated in oral cancer and melanoma cells (21,36). In the present study,



only low level of methylation could be detected in L9981 and NL9980 cells by bisulfite sequencing, while the MSP assay demonstrated non-methylation of KAI1 promoter in these two cell lines. There were reports from several groups suggested that methylation may not be the key mechanism for reduction of KAI1 expression, which suggest that other pathway was involved in KAI1 regulation (21,37,38). Taken together, these result implicated that KAI1 was not methylated at least in cancer cell NL9980 or L9981. Though we found KAI1 expression can be up-regulated by 5-AZA-dc, this might be an indirect effect.

In conclusion, the results of present study confirmed the suppressor activity of KAI1 in high metastasis potential L9981 cells, as well as suggested the positive relationship between KAI1 and nm23-H1 in lung cancer. We also showed that KAI1 promoter was activated by nm23-H1 in a p53-independent fashion. There should be more research aim to further analyze the interaction between KAI1 and nm23-H1. Moreover, the mechanism of synergistic inhibition of cancer metastasis by KAI1 and other metastasis suppressors is worth studying. If such a crosstalk was discovered between KAI1 signaling and pathways of other metastasis suppressors, new targets will be identified that helpful to the cancer therapy.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

### References

1. Travis WD. Pathology of lung cancer. *Clin Chest Med* 2002;23:65-81, viii.
2. Wang X, Ling C, Bai Y, et al. MicroRNA-206 is associated with invasion and metastasis of lung cancer. *Anat Rec (Hoboken)* 2011;294:88-92.
3. Marino N, Marshall JC, Steeg PS. Protein-protein interactions: a mechanism regulating the anti-metastatic properties of Nm23-H1. *Naunyn Schmiedebergs Arch Pharmacol* 2011;384:351-62.
4. Saha A, Robertson ES. Functional modulation of the metastatic suppressor Nm23-H1 by oncogenic viruses. *FEBS Lett* 2011;585:3174-84.
5. Ichikawa T, Ichikawa Y, Isaacs JT. Genetic factors and suppression of metastatic ability of prostatic cancer. *Cancer Res* 1991;51:3788-92.
6. Lee HA, Park I, Byun HJ, et al. Metastasis suppressor KAI1/CD82 attenuates the matrix adhesion of human prostate cancer cells by suppressing fibronectin expression and  $\beta$ 1 integrin activation. *Cell Physiol Biochem* 2011;27:575-86.
7. Tonoli H, Barrett JC. CD82 metastasis suppressor gene: a potential target for new therapeutics? *Trends Mol Med* 2005;11:563-70.
8. Dong JT, Isaacs WB, Barrett JC, et al. Genomic organization of the human KAI1 metastasis-suppressor gene. *Genomics* 1997;41:25-32.
9. Jee B, Jin K, Hahn JH, et al. Metastasis-suppressor KAI1/CD82 induces homotypic aggregation of human prostate cancer cells through Src-dependent pathway. *Exp Mol Med* 2003;35:30-7.
10. Bari R, Zhang YH, Zhang F, et al. Transmembrane interactions are needed for KAI1/CD82-mediated suppression of cancer invasion and metastasis. *Am J Pathol* 2009;174:647-60.
11. Upheber S, Karle A, Miller J, et al. Alternative splicing of KAI1 abrogates its tumor-suppressive effects on integrin  $\alpha\beta$ 3-mediated ovarian cancer biology. *Cell Signal* 2015;27:652-62.
12. Grybos A, Bar J. The relationships between the immunoeexpression of KAI1, MMP-2, MMP-9 and steroid receptors expression in endometrial cancer. *Folia Histochem Cytobiol* 2014;52:187-94.
13. Yu G, Bing Y, Li W, et al. Hepatitis B virus inhibits the expression of CD82 through hypermethylation of its promoter in hepatoma cells. *Mol Med Rep* 2014;10:2580-6.
14. Zhang W, Zhao CG, Sun HY, et al. Expression characteristics of KAI1 and vascular endothelial growth factor and their diagnostic value for hepatocellular carcinoma. *Gut Liver* 2014;8:536-42.
15. Yang CH, Chou HC, Fu YN, et al. EGFR over-expression in non-small cell lung cancers harboring EGFR mutations is associated with marked down-regulation of CD82. *Biochim Biophys Acta* 2015;1852:1540-9.
16. Mine M, Yamaguchi K, Sugiura T, et al. miR-203 Inhibits Frizzled-2 Expression via CD82/KAI1

- Expression in Human Lung Carcinoma Cells. *PLoS One* 2015;10:e0131350.
17. Zhou L, Yu L, Wu S, et al. Clinicopathological significance of KAI1 expression and epithelial-mesenchymal transition in non-small cell lung cancer. *World J Surg Oncol* 2015;13:234.
  18. Zhou Q, Wang Y, Che G, et al. Establishment and their biological characteristics of clonal cell subpopulations (NL9980 and L9981) from a human lung large cell carcinoma cell line (WCQH-9801). *Zhongguo Fei Ai Za Zhi* 2003;6:464-8.
  19. Che L, Zhou Q, Wang Y, et al. Establishment of a human large cell lung cancer cell line L9981-nm23-H1. *Zhongguo Fei Ai Za Zhi* 2004;7:187-90.
  20. Liu FS, Chen JT, Dong JT, et al. KAI1 metastasis suppressor gene is frequently down-regulated in cervical carcinoma. *Am J Pathol* 2001;159:1629-34.
  21. Kim YI, Shin MK, Lee JW, et al. Decreased expression of KAI1/CD82 metastasis suppressor gene is associated with loss of heterozygosity in melanoma cell lines. *Oncol Rep* 2009;21:159-64.
  22. Drucker L, Tohami T, Tartakover-Matalon S, et al. Promoter hypermethylation of tetraspanin members contributes to their silencing in myeloma cell lines. *Carcinogenesis* 2006;27:197-204.
  23. Mashimo T, Watabe M, Hirota S, et al. The expression of the KAI1 gene, a tumor metastasis suppressor, is directly activated by p53. *Proc Natl Acad Sci U S A* 1998;95:11307-11.
  24. Moez S, Malik FA, Kayani MA, et al. Expressional alterations and transcript isoforms of metastasis suppressor genes (KAI1 and KiSS1) in breast cancer patients. *Asian Pac J Cancer Prev* 2011;12:2785-91.
  25. Shiwu WU, Lan Y, Wenqing S, et al. Expression and clinical significance of CD82/KAI1 and E-cadherin in non-small cell lung cancer. *Arch Iran Med* 2012;15:707-12.
  26. Park JJ, Jin YB, Lee YJ, et al. KAI1 suppresses HIF-1 $\alpha$  and VEGF expression by blocking CDCP1-enhanced Src activation in prostate cancer. *BMC Cancer* 2012;12:81.
  27. Rowe A, Weiske J, Kramer TS, et al. Phorbol ester enhances KAI1 transcription by recruiting Tip60/Pontin complexes. *Neoplasia* 2008;10:1421-32, following 1432.
  28. Goncharuk VN, del-Rosario A, Kren L, et al. Co-downregulation of PTEN, KAI-1, and nm23-H1 tumor/metastasis suppressor proteins in non-small cell lung cancer. *Ann Diagn Pathol* 2004;8:6-16.
  29. Bozdogan O, Yulug IG, Vargel I, et al. Differential expression patterns of metastasis suppressor proteins in basal cell carcinoma. *Int J Dermatol* 2015;54:905-15.
  30. Friess H, Guo XZ, Tempia-Caliera AA, et al. Differential expression of metastasis-associated genes in papilla of vater and pancreatic cancer correlates with disease stage. *J Clin Oncol* 2001;19:2422-32.
  31. Jiang Z, Wang X. Expression and significance of PTEN and nm23-H1 in the metastasis of non-small cell lung cancer. *Zhongguo Fei Ai Za Zhi* 2008;11:793-7.
  32. Tee YT, Chen GD, Lin LY, et al. Nm23-H1: a metastasis-associated gene. *Taiwan J Obstet Gynecol* 2006;45:107-13.
  33. Marreiros A, Czolij R, Yardley G, et al. Identification of regulatory regions within the KAI1 promoter: a role for binding of AP1, AP2 and p53. *Gene* 2003;302:155-64.
  34. Jung H, Seong HA, Ha H. NM23-H1 tumor suppressor and its interacting partner STRAP activate p53 function. *J Biol Chem* 2007;282:35293-307.
  35. Marreiros A, Dudgeon K, Dao V, et al. KAI1 promoter activity is dependent on p53, junB and AP2: evidence for a possible mechanism underlying loss of KAI1 expression in cancer cells. *Oncogene* 2005;24:637-49.
  36. Uzawa K, Ono K, Suzuki H, et al. High prevalence of decreased expression of KAI1 metastasis suppressor in human oral carcinogenesis. *Clin Cancer Res* 2002;8:828-35.
  37. Jackson P, Millar D, Kingsley E, et al. Methylation of a CpG island within the promoter region of the KAI1 metastasis suppressor gene is not responsible for down-regulation of KAI1 expression in invasive cancers or cancer cell lines. *Cancer Lett* 2000;157:169-76.
  38. Sekita N, Suzuki H, Ichikawa T, et al. Epigenetic regulation of the KAI1 metastasis suppressor gene in human prostate cancer cell lines. *Jpn J Cancer Res* 2001;92:947-51.

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