・Basic Research・

Effects of tumor metastasis suppressor gene nm23-H1 on invasion and proliferation of cervical cancer cell lines

Yan Yang，Shi Lu，Min-Fang Li and Ze-Hua Wang

Department of Obstetrics and Gynecology,
Union Hospital,
Tongji Medical College,
Huazhong University of Science and Technology,
Wuhan，*Hubei*，*430022*
P. R. China

Correspondence to：Ze-Hua Wang
Tel.：86.27.85351685
Fax：86.27.85351685
E-mail：zehuawang@163.net

［Abstract］ Background and Objective： Previous studies have shown that nm23-H1 is a tumor metastasis suppressor gene. Nucleotide diphosphate kinase 1 （NDPK1） encoded by nm23-H1 is involved in cancer cellular differentiation, proliferation, apoptosis and metastasis. This study was to investigate the effects of nm23-H1 on proliferation and invasion of cervical cancer cells. Methods： The eukaryotic expression vector pcDNA3.1-nm23-H1 was transfected into cervical cancer cells. Cell invasion potential was determined by the Transwell assay. Cell proliferation was measured by MTT assay and changes in cell cycle distribution were analyzed by flow cytometry （FCM）. Results： Compared with parent cells （Caski and SiHa） and vector control cells （Caski-3.1 and SiHa-3.1）, the proliferation and invasion of pcDNA3.1-nm23-H1 transfected cells （SiHa-N and Caski-N） were apparently decreased （$P<0.05$）； the proportions of G_2/M and S cells were obviously decreased while that of G_0/G_1 cells was increased （$P<0.05$）. However, transfection of nm23-H1 gene had no influence on proliferation, cell cycle and invasion of HeLa cells （$P>0.05$）. Conclusion： nm23-H1 gene could inhibit proliferation and invasion of cervical cancer cells in a cell-dependent manner.
Key words： nm23-H1，transfect，cervical cancer，proliferation，invasion，cell cycle

Cervical cancer is the most common gynecologic malignant tumor, lymph node metastases and local invasion are high risk factors leading to poor prognosis.[1] Low expression or mutations of anti-metastasis gene non-metastasis 23-H1 （nm23- H1）, which associate with proliferation, invasion, migration and differentiation of tumor cells, is closely related to neoplasm metastasis.[2] When being transfected into human breast cancer cells, the eukaryotic expression vector nm23-H1cDNA can inhibit the proliferation and metastasis of tumor cells.[2-3] However, we still have little knowledge about the correlation of nm23-H1 to proliferation and metastases of cervical cancer cells. This study was to investigate the effects of nm23-H1 on proliferation and invasion of cervical cancer cells.

Materials and Methods

Materials. The following materials were used: cervical cancer cell lines HeLa, SiHa, Caski (China Center for Type Culture Collection), PcDNA3.1 nm23-H1 eukaryotic expression vector and plasmid pcDNA3.1 (presented by Dr. Xiong of Guangzhou Jinan University) DMEM-HD, fetal calf serum (FCS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Gibco). Trizol, Lipofectamine™ 2000 (Invitrogen). RevertAid First Strand cDNA Synthesis Kit (Fermentas), Taq enzyme, RNA enzyme inhibitors and dNTP (TOYOBO), protein extracted kit (Pulilai). Transwell (Corning), Matrigel™ Basement Membrane Matrix (Bioscience BD).

Methods. <u>Cell culture and transfection.</u> Human cervical cancer cell lines HeLa, SiHa and Caski were suspended in DMEM supplemented with 10% FCS, and cultured at 37° C in humidied atmosphere with 5% CO2. pcDNA3.1nm23-H1 and pcDNA3.1 plasmid was transfected into cells in the exponential phase of growth, respectively, using Lipofectamine™ 2000. G418 (HeLa: 600μ g/mL, SiHa: 700μ g/mL, Caski: 700μ g/mL) was added and maintained at a concentration of 300μ g/mL. The three treatment groups were named: HeLa-N, SiHa-N, Caski-N; the vector control groups were named HeLa-3.1,SiHa-3.1,Caski-3.1.

<u>The expression of nm23-H1 mRNA</u>. The expression of nm23-H1 in cervical cancer cells was detected by semi-quantitative RTPCR, using β -actin as the internal control. The primer sequences of nm23-H1 gene were as follows: sense, 5′ -ATGGC2CAACTGTGAGCGTACC-3′ ; antisense, 5′ -TCATTCATAGATCCAGTTCTG-3′ , 460bp. The primer sequences of β -actin were: sense, 5-CGGCATCGTCACCAACTG-3; antisense, 5-GGAAGGTGGACAGCGAGG -3, 839bp. PCR conditions were as follows: 60s at 95° C, 30s at 95° C, 35s at 57° C, and 30s at 72° C, in total 35 cycles, followed by a 5min extension at 72° C. PCR products (3μ L each) were analyzed by electrophoresis on a 1.5% agarose gel. The expression level of nm23-H1 mRNA was detected using the optical density value of nm23-H1 / β -actin. All experiments were performed in triplicate.

<u>The expression of nm23-H1 protein</u>. The protein level of nm23-H1 in cervical cancer cells was determined by western blot, using β -actin as the internal control. The total protein was extracted according to the specification. Protein samples (50 ug) were electrophoresed with a sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes. The anti-nm23-H1 antibody was used to determine nm23-H1 protein expression. The image analyzer and KDS ID 3.0.1 software were used to quantify the protein levels. All experiments were performed in triplicate.

<u>MTT assay</u> The proliferation capacity of tumor cells was measured by MTT assay. Cells were plated at 1×10^3 per 100 μ l in 96-well plates, and each cell line had three parallel wells. Then, 10μ L MTT (5g/L) was added to each well every 24h. After 4h incubation, DMSO 150μ L was added into each well, and the plate was oscillated for 10min. Enzyme-linked immunoassay was performed every day for six days to detect the absorbance at 570nm (A_{570}). Finally, the growth cure was plotted. The experiments were performed in triplicate.

<u>Flow cytometry</u> Cells were detached from the ask using 0.25% trypsin-EDTA. Cells were suspended into complete medium, and washed three times with PBS (1000r/min, 5min), then resuspended with 100μ L PBS and 300μ L PI stains containing RNA enzyme. All incubations were performed for 15 min before performing flow cytometry. Data were collected and analyzed using CellQuest software.

<u>Transwell assay</u> Cell invasive potential was determined by Transwell assay. The epi chamber of the Transwell was wrapped with 50mg/L matrigel glue dilution (1:8), and placed at 4℃ drying overnight. In the following day, the residue was removed, 50μ L serum-free medium supplemented with 10g/L bovine serum albumin (BSA) was added, and incubated at 37℃ for 30min. Before the experiment, cells were

cultured in serum-free media overnight, and resuspended in serum-free medium supplemented with BSA. The epi chamber of the Transwell was filled with 200μL cell suspension, the other chamber was inoculated with the supernatant of NIH3T3 cells. Each group had three parallel wells. Sixteen hours later, cells were stained by trypan blue, and counted by 200x light microscopy. All experiments were performed in triplicate.

Statistical analysis. All data represent the means of three independent experimental samples, expressed by . Statistical significance was determined using the students t-test. A p value of <0.05 was considered significant.

Results

The expression level of nm23-H1 mRNA in different cell lines. Expression levels of nm23-H1 mRNA (460bp) in HeLa-N (0.73± 0.00), SiHa-N (0.60± 0.01) and Caski-N (0.66± 0.01) were significantly higher than those in the corresponding parent cell lines HeLa (0.21± 0.01), SiHa (0.19± 0.01) and Caski (0.00± 0.00) and those in vector control cells HeLa-3.1 (0.23± 0.010), SiHa-3.1 (0.19± 0.01) and Caski-3.1 (0.00± 0.00) (p <0.05). The expression levels of nm23-H1 mRNA in vector control cells were not significantly different with those in parent cell lines (p> 0.05), suggesting that the vector did not obviously influence the expression of nm23-H1 mRNA (Fig. 1).

The expression level of nm23-H1 protein in different cell lines. The expression levels of nm23-H1 protein (17ku) in HeLa-N (0.34± 0.00), SiHa-N (0.48± 0.01) and Caski-N
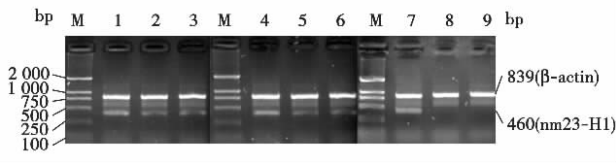

Figure 1　mRNA expression levels of nm23-H1 gene in cervical cancer cells before and after transfection
Lane M：DL-2000 marker；Lane 1：HeLa-N；Lane 2：HeLa-3.1；Lane 3：HeLa；Lane 4：SiHa-N；Lane 5：SiHa-3.1；Lane 6：SiHa；Lane 7：Caski-N；Lane 8：Caski-3.1；Lane 9：Caski.

(0.51± 0.01) were significantly stronger than those in the corresponding parent cell lines HeLa (0.06± 0.00), SiHa (0.07± 0.00) and Caski (0.01± 0.00) and those in vector control cells HeLa-3.1 (0.05± 0.00), SiHa-3.1 (0.08± 0.00) and Caski-3.1 (0.01± 0.00) (p <0.05). There was no difference in the protein expression of nm23-H1 among parent cells and vector control cells (p >0.05) (Fig 2).
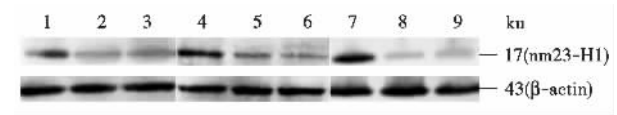

Figure 2　Protein expression levels of nm23-H1 in cervical cancer cells before and after transfection
Lane 1：HeLa-N；Lane 2：HeLa-3.1；Lane 3：HeLa；Lane 4：SiHa-N；Lane 5：SiHa-3.1；Lane 6：SiHa；Lane 7：Caski-N；Lane 8：Caski-3.1；Lane 9：Caski.

The effect of nm23-H1 on proliferation of cervical cancer cells. Three days after transfection, the proliferation level of SiHa-N cells was significantly lower than that of SiHa-3.1 cells and SiHa cells [(0.46± 0.01) vs. (1.32± 0.01) and (1.33± 0.01)] (p <0.05). On the forth-sixth day, A_{570} of SiHa-N cells was significantly lower than that of SiHa-3.1 and SiHa cells (p<0.05). On the third day, the proliferation level of Caski-N cells was significantly lower than that of Caski-3.1 and Caski cells [(0.33± 0.02) vs. (0.61± 0.02) and (0.61± 0.02)] (p < 0.05), such differences still existed three days later (p < 0.05). However, no significant difference in proliferation was found among HeLa-N, HeLa and HeLa-3.1 cells [(1.10 ± 0.01) vs. (1.15 ± 0.01) and (1.18 ± 0.02)] (p > 0.05) (Fig. 3).

The effect of nm23-H1 on the cell cycle of cervical cancer cells. Compared with SiHa and Siha-3.1 cells, the proportion of SiHa-N cells in G_0/G_1 phase was increased (p < 0.05), while cells in G_2/M and S phases were obviously decreased (p < 0.05); nm23-H1 exerted a similar effect on Caski cells (p <0.05), but had no influence on HeLa cells (p>0.05) (Table 1).

The effect of nm23-H1 on the invasive ability of cervical cancer cells. Compared with Caski-3.1 (88.00 ± 4.00) and Caski cells
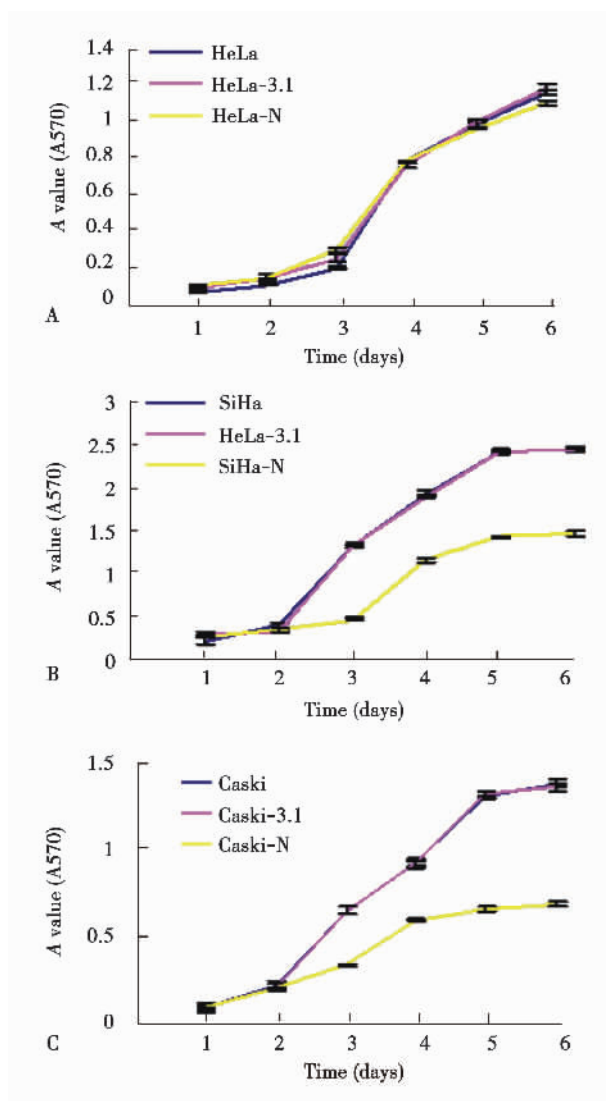
Figure 3   Effects of nm23-H1 on proliferation of different cervical cancer cells

All values are presented as mean ±SD of three independent experiments.

Table 1   Effects of nm23-H1 on cell cycle distribution of different cervical cancer cells

Cell line	Cell cycle proportion（%, $\bar{x} \pm s$）		
	G_0/G_1	S	G_2/M
HeLa	65.42±0.60	18.21±0.74	16.60±0.37
HeLa-3.1	65.83±0.97	18.41±0.51	17.34±0.47
HeLa-N	65.30±0.62	17.58±0.06	17.31±0.66
SiHa	47.31±0.33	36.32±0.23	16.34±0.34
SiHa-3.1	47.66±0.11	36.27±0.64	16.33±0.45
SiHa-N	60.16±0.63	25.46±1.07	11.08±0.12
Caski	30.62±0.40	32.58±0.60	37.62±0.45
Caski-3.1	31.13±0.83	29.65±0.37	37.41±0.52
Caski-N	56.35±0.39	21.42±0.54	22.72±0.27

All values are presented as mean ±SD of three independent experiments.

(86.67± 3.46), the average number of Caki-N cells permeating the Transwell membrane was much less (p<0.05). The average number of SiHa-N cells (18.33 ± 2.5) permeating the Transwell membrane was also less than that of SiHa-3.1 (100.33 ± 1.52) and SiHa cells (99.67± 2.08) (p < 0.05). However, there was no statistical significance in cell invasion among HeLa (89.21± 1.89), HeLa-3.1 (88.87± 1.73) and HeLa-N (89.72± 1.15) cells (p > 0.05) (Fig 4).

Discussion

Occurrence and development of diffusion and metastasis is a complex process, involving the low expression, deletion or malfunction of anti-oncogenes and overexpression or malfunction of oncogenes. Recently, nm23-H1, an anti-metastasis gene, has been reported to be correlated with the occurrence and development of cervical cancer. Luo et al.[4] detected 69 cases of cervical cancer tissues and 20 cases of normal cervical tissues, she found that the positive expression of nm23 has negative correlation with clinical stage, pathology classification and lymph node metastasis of cervical cancer (p < 0.01), and that the deletion of nm23 plays an important role in the occurrence, development and metastasis of cervical cancer. However, the research of Utrera-Barillas et al.[5] indicated that nm23-H1 could adjust invasion and metastasis of cervical cancer, but only in the cases with high homocysteine protease vitality; the survival rate of patient with positive expression of nm23-H1 was obviously higher than that in patients with negative expression (p < 0.01). However, nm23-H1 can not be the independent prognostic indicator of cervical cancer. nm23-H1 has a complex relationship with H-ras and proteolytic enzymes, which influences the clinical features of tumors. Thus, the correlation between nm23-H1 and cervical cancer tissues is complicated and modulated by various factors.

In this study, wild type nm23-H1 was transferred into different cervical cancer cell lines the results confirmed that HeLa-N, SiHa-N and Caski-N cell lines were highly expressed
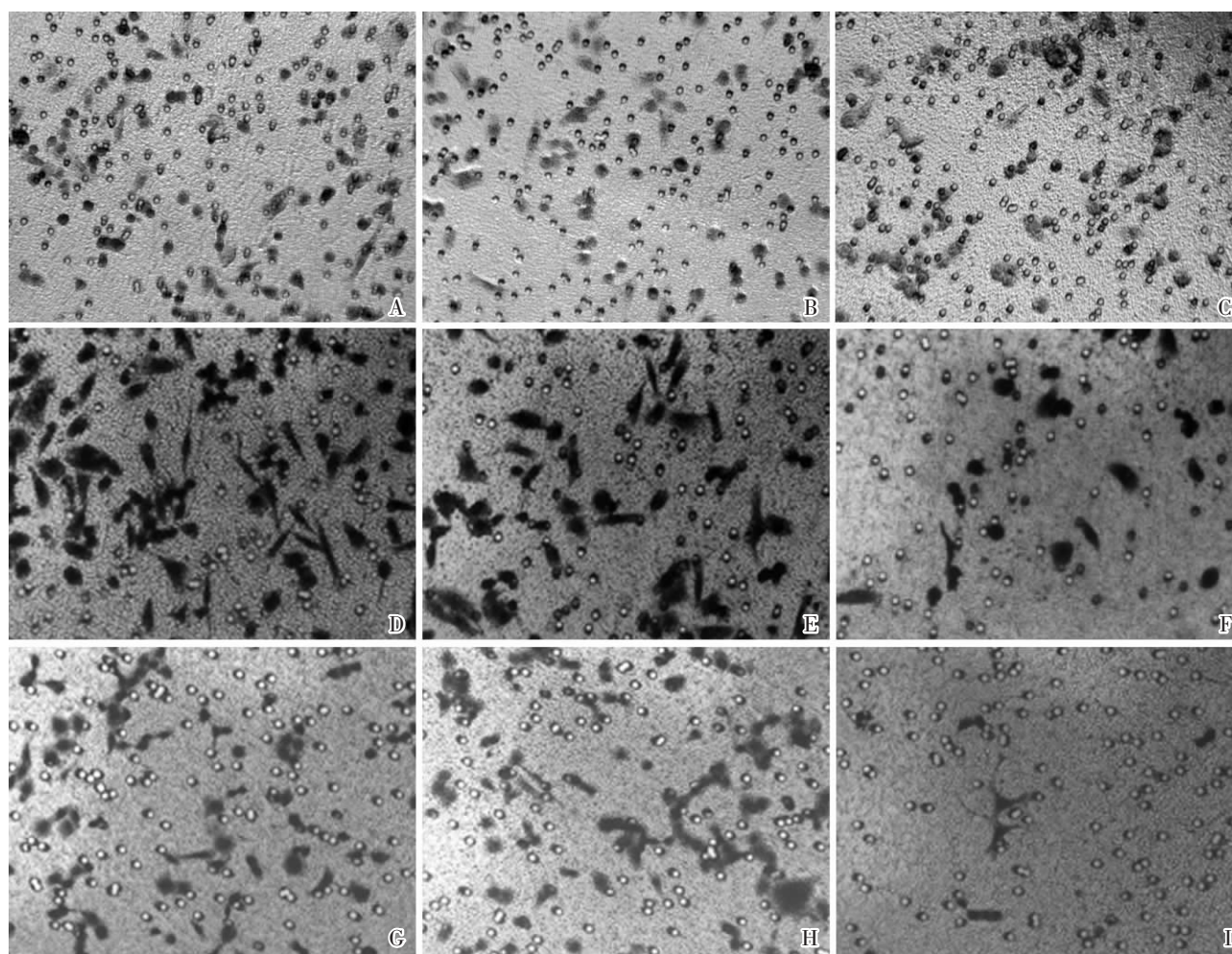
Figure 4   Effects of nm23-H1 on invasive capacity of different cervical cancer cells
A：HeLa；B：HeLa-3.1；C：HeLa-N；D：SiHa；E：SiHa-3.1；F：SiHa-N；G：Caski；H：Caski-3.1；I：Caski-N.

nm23-H1. Moreover, nm23-H1 obviously inhibited the proliferation and invasion potential of SiHa and Caski cells. These demonstrated that nm23-H1 could reverse the phenotype which determines the proliferation, invasion and metastasis of cervical cancer, and it could be highly expressed in the cervical cancer cell lines through stable transfection. Our results were in line with other studies.[6,7]

Tumor spread is a multifactorial process, and nm23-H1 regulates multiple links of this process. It can reduce the motorcity, invasion and proliferation of the tumor cells, and promote cell differentiation at the same time.[2] Currently, it is indicated that, in squamous carcinoma of the cervix tissues, the high activity of cathepsin B1 is obviously relevant with the deletion of nm23-H1, which indicates poor prognosis.[5] The

combination of the carboxyl terminus of Rad protein and calmodulin (CaM) could promote the conversion of CaM from G1 phase to S phase. Once nm23-H1 is combined with Rad, it can inhibit Rad and reduce cell proliferation through regulating the cell cycle.[8] We confirmed that nm23-H1 can obviously decrease the proportions of SiHa-N and Caski-N cells in G_2/M and S phases, elevate the proportion of cells at G_0 / G1 phase. Thus, nm23-H1interferences the malignant proliferation of tumor cells through adjusting the cell cycle, which may be related to its interaction with Rad protein.

Nm23-H1 is a kind of pleiotropic gene, and it has different regulatory mechanisms in different tumor cells. It is indicated that oncogene N-myc could up-regulate the

expression of nm23-H1 protein and inhibit cell differentiation in neuroblastoma, thus, to increase the invasiveness of tumor cells.[9] Different tumor cell lines have different gene expression profiling and different biological behaviors. It is also confirmed that the gene expression profilings of adenocarcinoma of the uterine cervix and squamous carcinoma of the cervix exist significant differences.[10] Compared with squamous cell carcinoma, adenocarcinoma possesses higher invasive phenotype, which manifests a higher metastatic rate and lower survival rate.[11] RT-PCR and Western blot results displayed that HeLa cells expressed a small amount of nm23-H1 mRNA and protein. After transfection with wild-type nm23-H1, nm23-H1 was highly expressed in HeLa cells, but its cell cycle, proliferation and invasiveness were not significantly altered. However, the proliferation and invasive capacity of Caski cells and SiHa cells were obviously restrained after the transfection. This suggests that the effects of nm23-H1 on cervical cancer cell lines are cell-specific. The mechanism needs to be further studied.

In summary, by changing cell cycle of tumor cells, nm23-H1 could inhibit the proliferation and invasiveness of Caski and SiHa cells. But it has no effect on the cell cycle, proliferation and invasiveness of Hela, an adenocarcinoma cell line of the cervix. nm23-H1 may influence the biological phenotype of tumor cells through different mechanisms, such as proliferation and invasiveness.

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