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Suppressing stathmin-1 can inhibit chk1 protein expression and reduce the invasion and tumorigenicity of cervical cancer cells

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Summary

The purpose of this study was to evaluate the effects of stathmin-1 on chk1 protein expression in cervical cancer cells and the influences on the cells' invasion and tumorigenicity. Suppressed stathmin-1 expression in Hela cells and C33A cells by lentiviral vector were utilized. Real time PCR and Western blot were used to examine the expression of chk1. Cell proliferation and invasion were studied using MTT assays and transwell migration assays.The differences of tumorigenicity in vivo were explored using xenograft experiments. In addition, stathmin-1 expressions in 24 cervical cancer patients were studied without regional lymph nodes metastasis and 16 metastatic patients by immunohistochemistry assays and real time PCR. This study found downregulating stathmin-1 reduced chk1 expressions and the proliferation and invasion in cervical cancer cells and reduced the tumorigenicity of tumor cells.

Key words: Stathmin-1; chk1; Cervical cancer; Invasion; Tumorigenicity.

Introduction

Stathmin is a ubiquitous microtubule-destabilizing protein shown to be important during mitosis and has been implicated as a regulator of cell motility and migration [1]. Stathmin has gained attention as prognositic biomarker for aggressive invasive and metastatic tumors from multiple organs such as bladder, lung, liver, breast, ovary, oral squamous [2-7], and others. Suppressing stathmin expression has been shown to decrease cellular proliferation and invasion in prostatic and gastric cancers [8, 9]. It is demonstrated that stathmin is involved in cell proliferation, cell cycle progression, apoptosis, and plays an essential role in the invasion and metastasis of cancer [1].

Cervical cancer is one of the most common gynaecologic malignancies around the world. It has been estimated to have been responsible for almost 260,000 deaths annually, of which about 80% occurred in developing countries [10]. Currently in China, about 135,000 new cases of cervical cancer occur each year, accounting for one-third of the number of global incidences [11]. Several reports have shown that stathmin expression increase is present in high-grade cervical precursor lesions and invasive carcinomas and higher levels of stathmin expression are correlated with a shorter survival time and poorer prognosis [12, 13].

Some studies have been conducted to investigate the changes of cancer cells with stathmin expression downregulation. Stathmin depletion leads to an accumulation of cells in the G2 cell cycle stage in Hela cells [14]. In nasopharyngeal carcinoma (NPC) cell silencing of stathmin suppresses proliferation, invasion, and metastasis, and induces apoptosis [15]. However, the mechanisms have not been defined. Chk1 is an upstream activator for CDC25 and targets the CDC25/WEE1-cyclinB1-CDK1 axis which is associated with stathmin activation [16, 17]. There is currently no data exploring the influences of stathmin suppressed on chk1 expression and cellular invasion and tumorigenicity in cervical cancer cells which formed the reason behind conducting the present investigation.

Materials and Methods

Cell culture

Hela Cells and C33A cells (donated from etiology Laboratory of Qingdao University Medical College) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 ug/ml penicillin and 100 ug/ml streptomycin and supplemented with 10% calf blood serum at 37°C in a humidified atmosphere with 5% CO2.

ShRNAs targeting to stathmin-1 was designed and constructed into hU6-MCS-CMV-EGFP. Four siRNA sequences from various coding regions of stathmin-1 gene were the following: stathmin-1-260, 5- CCCATGAAGCTGAGGTCTT -3 (from 260 bp), named as S1; stathmin-1-241, 5- GCAGAAGAAAGACGCAAGT -3 (from 241 bp), named S2; stathmin-1-430, 5- CTGGAACGTTTGCGAGAGA -3 (from 430 bp), named S3; stathmin-1-303, and 5- GCACGAGAAAGAAGTGCTT -3 (from 303 bp), named S4. Control sequences: 5- TTCTCCGAACGTGTCACGT-3. Then shRNA plas-

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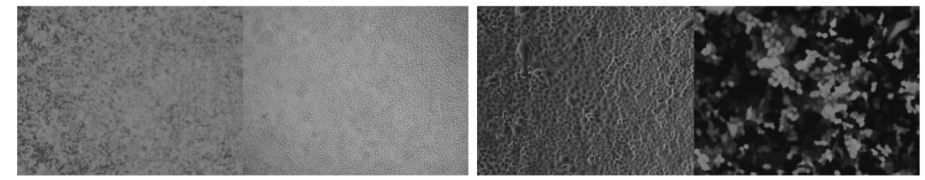
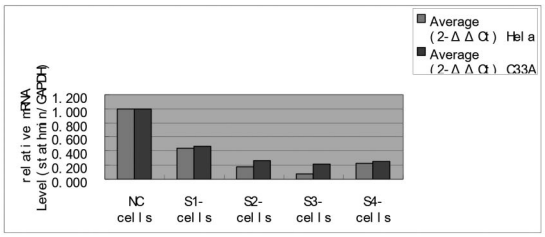
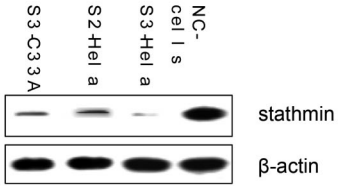


A 293Tcells transfected and GFP expr ession in cells

B Hela cells infected with lentirus an d GFP expression



C The relative mRNA Level(stathmin/GAPDH)in cells i nfected by recombinant lentivirus carrying different shR NA(S1-S4)



D stathmin-1 protein exp ression in Hela and C33 Acells

Figure 1. — S1-S4 could all reduce the expression of stathmin-1mRNA in cells, and S3 had the most obvious inhibition, as shown in C($P<0.05$); Western-blot assay had consistent result as shown in D($P<0.05$).

mids named si-stathmin-1 and control plasmids named conlentivirus were identified using enzyme digested, further confirmed by direct sequencing according to the manufacturer's protocol. Recombined Lentiviral plasmids were transfected into HEK293T cells for packing lentivirus.

Cells infection studies were carried out with 3×10^4 cells/ml plated on a 12-well plate (one ml/well). Recombinant virus infected Hela cells (MOI=5) and C33A cells (MOI=50) and GFP were explored 72 hours after infection. Total RNA was isolated from cells using the TRIzol reagent according to the manufacturer's instructions. The expression of stathmin-1 in infected cells was assessed by real-time PCR analysis. Primers used in these studies are indicated here: GAPDH (TGACTTCAACAGCGACACCCA, CACCCT GTTGCTGTAGCCAAA, 121 bp in total); stathmin-1 (TGAGAAA CG AGAGCACGAG, TCAGCAGGGTCTTTGGATTC, 213 bp in total).

MTT assays: cells were plated on 96-well plates and were infected by recombinant lentivirus. The 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazoliumbromide (MTT) assay was used to evaluate cell proliferation at 0, 24, 48, 72, and 96 hours. The spectrophotometric absorbance of each sample was measured at 490 nm.

Migration assays:1×10^5 cervical cancer cells were plated in the top chamber with a non-coated membrane (24-well insert; eight-um pore size). The cells were plated in serum-free medium, and medium supplemented with 20% serum was used as a chemoattractant in the lower chamber. The cells were incubated for 16 hours at 37°C and 5% CO2 in a tissue culture incubator. After 16 hours, the non-migrated cells were removed from the upper sides of the transwell membrane filter inserts using cotton-tipped swabs. For the invasion assays, 1×10^5 cervical cancer cells were plated in the top chamber with a matrigel-coated membrane (24-well insert; eight-um pore size. The other experimental procedures were same as migration assays. For both assays, the migrated/invaded cells on the lower sides of the inserts were stained with crystal violet. Then the dye was cleaned with 33% acetic acid and the spectrophotometric absorbance of each sample was measured at 570 nm.

Recombinant virus infected cells and GFP were explored 72 hours after infection. Cells were lysed and proteins were extracted. Protein lysates were separated by 10% SDS-PAGE, transferred onto PVDF membrane, after blocking with 5% nonfat dry milk 2h, probed with specific antibodies (1:1000 for β-action, 1:1000 for chk1, 1:2000 for stathmin-1) overnight at 4°C, and revealed using the incubated with horseradish peroxidase-conjugated secondary antibody (1:4000) for two hours. They were then developed with an enhanced chemiluminescence Western blot detection kit and exposed to X-ray film.

1×10^6Hela cells with stathmin-1 suppressed and equal numbers of control Hela cells which were suspended in 100 ul PBS were injected subcutaneously into the right rear flank of each mouse (five mice per group) and tumor growth was monitored every three days in each group. The tumor volume was calculated using the formula $V=\text{longest dimension}\times(\text{shortest dimension})^2/2$. The mice were sacrificed 15 days later.

After approval of the Research Ethics Committee and the patients' informed consent, 16 cervical cancer patients with regional lymph nodes metastasis and 24 patients without metastasis were collected from 2012 to 2014 in Qingdao Hiser Medical Hospital. All the cervical cancer samples and lymph nodes were confirmed by pathological analysis. The RNA from tissue samples was isolated using TRIzol reagent following the manufacturer's instructions. The inspections of stathmin-1 in tissues were performed by immunohistochemical study, using antibodies to stathmin-1 (1:300 dilution). The staining result was assessed using positive cells and intensity.

Results

Four different ShRNA fragments targeting stathmin-1 were packaged in 293T cells and recombinant lentivirus infected Hela cells and C33A cells (Figure 1A, B) GFP expression could be investigated with the fluorescence microscope. Reduction of stathmin-1 mRNA expression was monitored by real-time PCR analysis, respectively. As shown

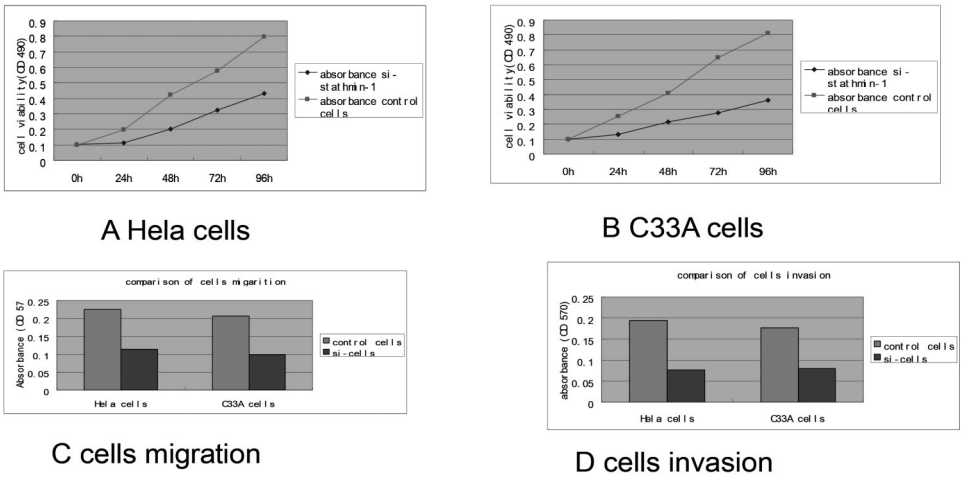


Figure 2. — The proliferation of Hela cells (A) and C33A cells (B) infected by si-stathmin lentivirus was significantly suppressed at 24, 48, 72 and 96 hours after infected compared to control cells ($P<0.05$); The results of migration (C) and invasion (D) showed that the numbers of si-stathmin-1 Hela and C33A cells crossing the transwell membranes were significantly ($p<0.001$) decreased compared to control group.

in Figures 1C, D, S3 caused more decrease of mRNA level of stathmin-1 than the other three shRNA ($p < 0.01$). Thus, the shRNA synthesized by S3 was used in the silencing system.

MTT assays showed the proliferation of Hela cells and C33A cells infected by si-stathmin lentivirus was significantly suppressed at 24, 48, 72, and 96 hours after infected compared to control cells infected with con-lentirus (Figures 2A, B) ($p < 0.05$). The results of migration examination revealed that the numbers of si-stathmin-1 lentivirus Hela and C33A cells crossing the transwell membranes were significantly ($p < 0.001$) decreased compared to either control group (Figure 2C), indicating that downregulation of stathmin-1 inhibits the migratory capacity of these cells. Similarly, this assay allowed only cells with an invasive capacity to cross the basement membrane layer. The invasion investigation results revealed that numbers of si-stathmin transfected cells that crossed the membrane layer was also significantly ($p < 0.001$) decreased compared to the number of control cells (Figure 2D). This latter result indicates that downregulation of stathmin expression also inhibits the invasive capacity of Hela and C33A cells.

Hela cells and C33A cells were infected by si-stathmin-1 lentivirus and con-lentivirus, and 72 hours later the GFP expressions in cells could be found. Then studied chk1 mRNA levels and the proteins expression by real time PCR and Western blot. The length of chk1 (primers 5-CGGTATAAT AATCGTGAGCG-3,5-TTCCAAGGGTTGAGGTATGT-3) fragments was 230 bp. The results showed that in both Hela cells and C33A cells infected by si-stathmin-1 lentivirus, the relative mRNA levels of chk1 and the protein expression were reduced obviously than in control cells infected by con-lentivirus (Figures 3A, B) ($p < 0.05$).

To study the influences of stathmin-1 on tumorigenesis, a xenograft tumor model was used in the severe combined immunodeficiency (SCID) mice. Human Hela cells in-

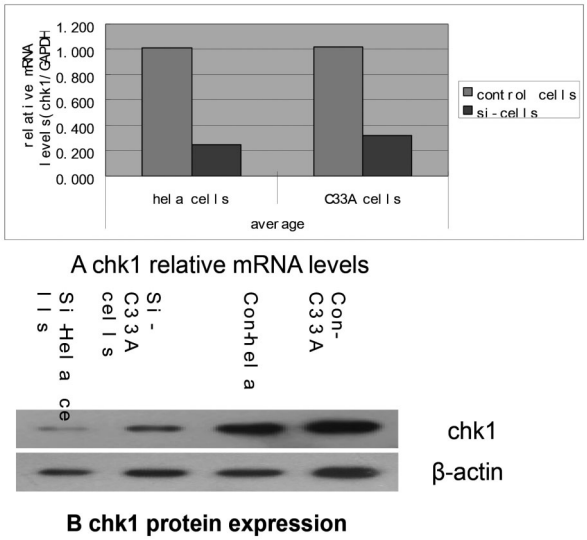


Figure 3. — In both Hela cells and C33A cells infected by si-stathmin-1 lentivirus the relative mRNA levels of chk1 (A) and the protein expression (B) were reduced obviously than in control cells infected by con-lentivirus ($P<0.05$).

fected with si-stathmin-1 lentivirus called si-Hela group and infected with con-lentivirus called con-Hela group were injected into nude mice. As a result, the volume of the tumors derived from the si-stathmin-1 Hela cells was dramatically reduced at 6, 9,12, and 15 days compared to the control group (Figure 4) ($p < 0.05$). The result is consistent with the proliferation examination of cultured cervical cancer cells. It indicated suppressing stathmin-1 could reduce the tumor cells growth and tumorigenicity in vivo.

Cervical patients with regional lymph nodes metastasis had

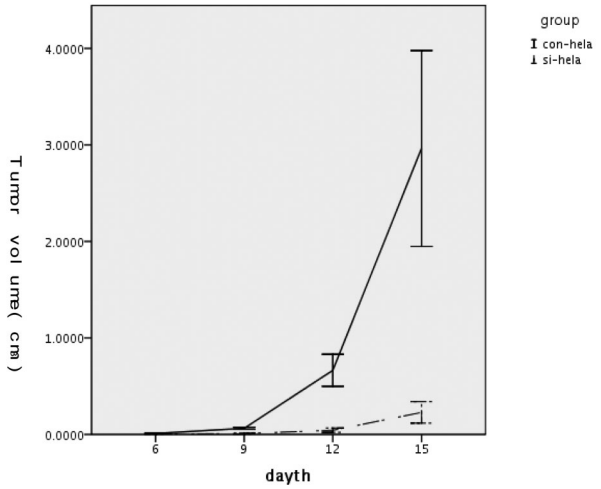


Figure 4. — The volume of the tumors derived from the si.stathmin-1 Hela (si-Hela) cells was dramatically reduced at 6, 9, 12 and 15 days compared to the control group (con-Hela) (P<0.05).

positive stathmin-1 expression in 93.8% and 70.8% patients without lymph nodes metastasis had stathmin-1 expression, but it had no significant difference ($p > 0.05$). However, the stathmin-1 mRNA levels had significant difference ($p < 0.05$) between the two group patients (Figure 5).

Discussion

Stathmin is seen as an oncoprotein; its overexpression and activity have been examined in several kinds of human cancer. Most of the studies show that it plays an important role in cell growth, motility, cancer onset and progression, and correlates with cancer cells increased proliferation, increased invasion, and with worse prognosis in several types of human cancer such as endometrial cancer [18, 19], brain [20], ovarian [21], oral cancer [22], and so on. In cervical cancers, studies have also shown that stathmin was correlate to clinicopathologic factors and poor prognosis. Some studies had shown suppressing stathmin could reduce the proliferation of tumor cells in vitro [23, 24]. In the present study the authors chose two kinds cervical cancer cells which had rich stathmin expression [25]. This two kinds of cells include HPV positive and HPV negative cells. The expression of stathmin-1 in these cells was suppressed by lentivirus-shRNA. Then the authors found that the proliferation of Hela cells and C33A cells was reduced significantly. In addition, they injected si-stathmin-1 hela cells and control hela cells in nude mice and explored the tumor cells proliferation in vivo. They found the xenografts of Hela cells suppressing stathmin-1 were obviously reduced than the xenografts of control Hela cells. The results about the influences of suppressing stathmin-1 expression on the proliferation of cervical cancers were consistent between the tests in vitro and in vivo. As we know that the tumor occurrence cannot be separated from the tumor cells excessive proliferation. This result proved the relationship be-

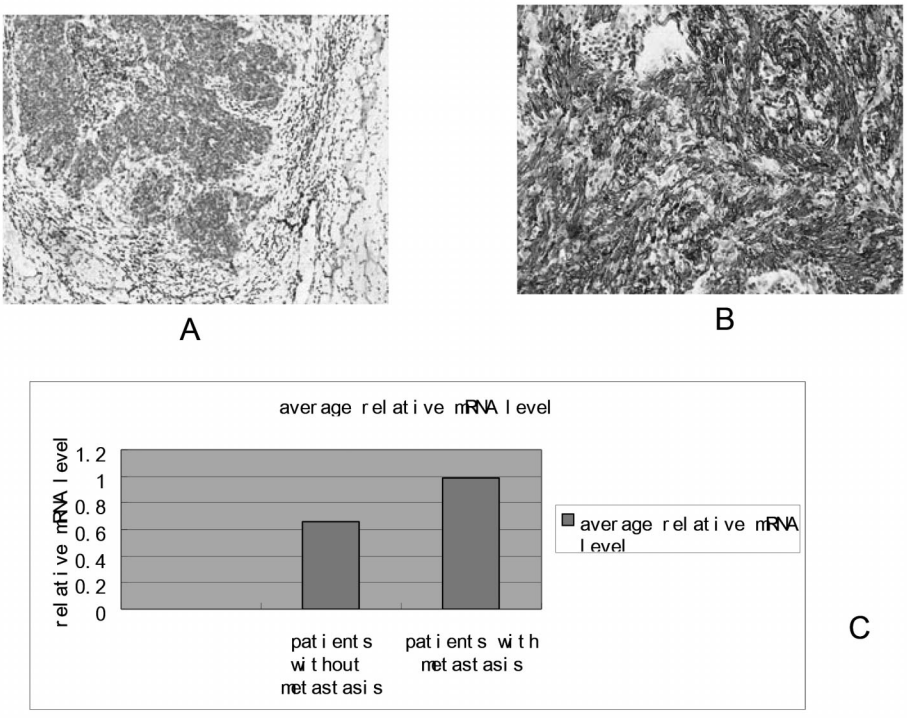


Figure 5. — Stathmin-1 positive expression was show in B. The stathmin-1 mRNA levels had significant difference (P<0.05) between the two group ptients as ahown in C.

tween stathmin-1 and cervical cancer cells tumorigenicity in vitro and in vivo. The study also investigated the changes of Hela cells and C33A cells invasion and migration when stathmin-1 knocked down these cells. The authors found suppressed expression of stathmin-1 inhibited invasion and migration obviously in Hela cells and C33A cells. The similar results had been reported in esophageal cells [23] and nasopharyngeal carcinoma cells [24]. In histological study of cervical cancers, the present authors found that the positive rates of stathmin-1 expression in cervical cancer patients with regional lymph nodes had a tendency to be higher than in patients that did had not have lymph nodes metastasis, but the difference was not statistically significant ($p > 0.05$). The stathmin-1 mRNA expression was higher in patients with regional lymph nodes metastasis than in patients without metastasis. No significant difference in positive rate may be related to the number of cases which was not enough. Tumor metastasis cannot be separated from the invasion and migration of tumor cells. The results indicated that stathmin-1 expression in cervical cancer was almost invariably associated with increased invasion and metastasis formation. One report has shown the correlation between stathmin protein expression and cervical cancer clinicopathological characters and the patients prognosis with immunohistochemistry [12]. In that investigation, the researchers studied the expression intensity of stathmin protein in different clinical stage patients and the results were consistent with the present.

Stathmin-1 expression and activity is related with many factors in proliferating cells. E2F has been proposed to be implicated in overexpression of stathmin in vivo in a mouse model of prostate cancer [26]. Recent data suggest that forkhead box (Fox) M1 is able to bind the stathmin promoter and to induce its expression in mammary carcinoma cell lines [27]. Wild type p53 is reported that it can restrain stathmin-1 activity [28]. Other researchers also reported that stathmin depletion reduced the activity of CDC25 and its upstream activators, aurora A and Plk1 [29]. Chk1 is also an upstream activator for CDC25 and targets the CDC25/WEE1-cyclinB1-CDK1 axis. Until now no evidences have shown the relationship of stathmin and chk1 protein expression. Chk1 is critical for cancer cell proliferation. To date, several cancer cells have been found to be Chk1 kinase-dependent such as neuroblastoma [30], melanoma [31], and so on. The study on cervical cancer cells has shown downregulation of Chk1 can kill Hela cells [32]. Previous studies have suggested that ChK1 and stathmin-1 were related to the development of tumor, and they may become a research point of tumor targeted therapy. However gene targeting therapy studies are facing many difficulties, such as safety, operability, and so on. Understanding more interactive mechanisms can provide new models and methods for the studies of targeted therapy. In the present study, the authors found downregulating stathmin-1 expression could reduced chk1 expression in Hela cells and C33A cells. These results indicated that in the processes of these cells cycle, the two factors may have synergies to cell cycle disorders and promote cell proliferation. Chk1 has been proved to be essential for tumorigenesis [33]. In cervical cancer cells, suppressing stathmin-1 expression could also reduced chk1, which may be one of the mechanisms in which inhibiting the expression of stathmin-1 could decrease cell oncogenicity. The present results provide novel ideas for more effective targeted therapy studies against cervical cancers.

In summary, suppressing stathmin-1 expression could reduce the proliferation, invasion, and migration in Hela and C33A cells. In vivo, downregulated stathmin-1 expression could suppress cancer cell tumorigenicity. Stathmin-1 expression was related with lymph nodes metastasis in cervical cancer patients which was consistent with cell experiments. Moreover, stathmin-1 depletion could reduced the expression of chk1 in Hela and C33A cells which may be one of the mechanisms of decreasing the cells oncogenicity. The investigation confirmed that stathmin expression was obviously correlated with more aggressive behavior of cervical cancer by the tests in vivo and in vitro, which provided the theoretical basis for future gene therapy research.

Acknowledgments

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