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Expression of cancer stem markers could be influenced by silencing of p16 gene in HeLa cervical carcinoma cells

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Summary

Effect of the tumor suppression gene p16 on the biological characteristics of HeLa cervical carcinoma cells was explored. The expression of p16 protein was increased in HeLa tumor sphere cells, and no significant difference in tumor spheres from the first to the fourth passages. Compared with those of parental HeLa cells, the proportion of CD44+/CD24- and ABCG2+ cells increased significantly in tumor spheres. However after the cells were silenced by the p16-sh289 vector, expression of P16 protein and the cell number of CD44+/CD24- and ABCG2+ decreased. Moreover, HeLa cells with p16 gene silencing showed decreased abilities of sphere formation and matrigel invasion. More HeLa cells with p16 gene silence were needed for tumor formation in nude mice. Tumor size and weight in mouse model established with p16 gene silenced HeLa cells were less than those with HeLa parental cell model. The present results indicate that silencing of the p16 gene inhibits expression of cancer stem cell markers and tumorigenic ability of HeLa cells.

Key words: p16; Gene silencing; HeLa cell; Cancer stem cell; shRNA; CD44; CD24; ABCG2.

Introduction

The p16 gene, also known as the $p16^{INK4A}$ gene, is well known as a tumor suppressor in various carcinomas. The frequent mutations, deletions, and gene methylation of the p16 gene are associated with various tumors. However, increased p16 gene expression in cervical squamous cell carcinoma has been detected. Expression of p16 protein has been shown to gradually increase with increasing grades of cervical cancer [1-4]. A recent study has indicated that p16 protein could be used as a biomarker in the diagnosis of human papillomavirus (HPV)-related cervical lesions [5].

The role of the p16 gene in the carcinogenesis of cervical cancer may be related with several mechanisms. The E7 protein of HPV induces overexpression of p16 by suppression of pRB to p16 [6, 7]. Dysfunction of p53 and pRB due to the integration of HPV E6 and E7 into the host cell chromosome results in increased expression of p16, causing cell cycle alteration in cervical epithelial cells and carcinogenesis progression. Overexpression of the p16 gene inhibits pRB function, thus increasing expression of the cyclin D-CDK complex, which stimulates P16 expression in a feedback manner [8].

Cancer stem cells are cancer cells that possess characteristics associated with normal stem cells, and are tumorigenic. They may generate tumors through the stem cell processes of self-renewal and differentiation. Such cells are proposed to persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors. Treatment resistance of cervical cancer is related to cervical cancer stem cell, which is confirmed by high expressions of cancer stem cell markers such as Oct3/4, CD133, and SOX2 [9-11]. Special effect of p16 gene in cervical carcinogenesis has been found because of its high expression in tumor cells. Little is known however, of the influence of p16 gene on the biological behavior of cervical cancer stem cells.

Materials and Methods

Cervical cancer cell lines and tumor sphere culture

The HeLa cervical cancer cell line (ATCC) was maintained in complete Dulbecco's Modified Eagles Medium (DMEM) following the manufacturers' instructions. The HeLa cells were cultured in sphere culture medium (serum free) to induce tumor cell spheres in low adhesion culture plate. For the first passage of tumor sphere culture, $1x10^4$ cancer cells were seeded into a six-well plate with sphere culture medium. Cells were cultured for about three to five days at 37ºC. When cell numbers in a single sphere reached 100-200, cells were harvested with a pipette and digested with 0.25% trypsin. Single cells were separated from spheres and transferred into a new cell culture flasks for future culture and experiment.

Transfection of HeLa cells with lentivirus-shRNA

The pLenti6/BLOCK-iT DEST vector system was used to construct p16-sh289 for silencing the gene expression. To construct a stable p16 gene silencing line, the HeLa parental cell line was transfected with p16-sh289 and screened by Western blot. The primer sequences of p16-sh289 were: 5'-caccgaccaggacctaaggacatatcgaaatatgtccttaggtcctggtc-3', 3'- ctggtcctggattcctgtatagctttatacaggaatccaggaccagaaaa-5'.

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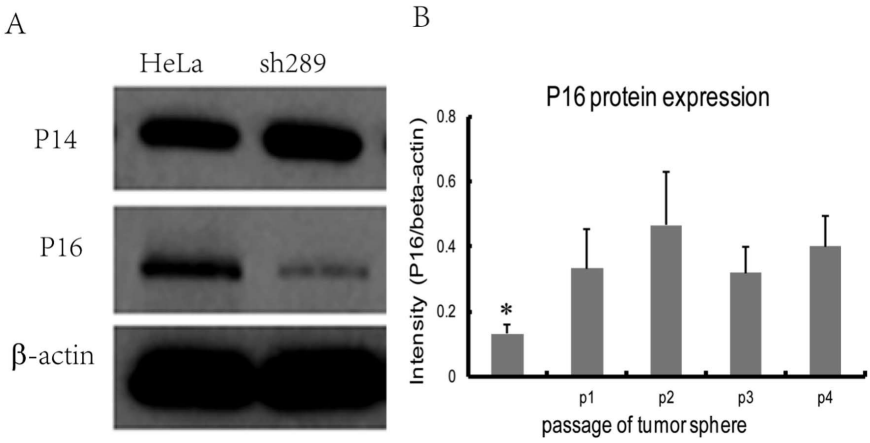

Figure 1. — p16 protein expression in HeLa parental cell line and sphere forming cells. A) P16 protein downregulated in HeLa cells after sh289 transfection. (p14 expression not influenced). B) Tumor spheres were cultured from parental HeLa cells. P16 protein expression in parental HeLa cells was less than those in tumor spheres (*$p < 0.05$). There was no difference among cells from passage 1 to passage 4. p1-p4 represents passages.

Flow cytometry analysis

Tumor spheres were digested with 0.25% trypsin for approximately three minutes, neutralized with medium containing serum, collected, and pipetted into single cell suspension. Following washing twice with PBS containing 1% BSA, the cells were incubated with the appropriate concentration of florescence conjugated primary antibodies for 45 minutes at 4°C. The cells were then washed with PBS×3. All samples were measured using a FACS Calibur flow cytometer and analyzed with CellQuest software. The antibodies used included FITC-CD44, PE-CD24 and FITC-ABCG2.

Western blot analysis of p16 protein expression

Total protein was extracted with lysis buffer, underwent PAGE electrophoresis and transferred to nitrocellulose membrane. The antibodies used in the current study included mouse anti-human p16 and p14 and anti-mouse IgG-HRP antibody. Nitrocellulose membrane was developed using the ECL method as the manual described.

Cell invasion assay in vitro

A total of 200 μL of 2×10^5/ml cells were added to insert chambers mounted on a six-well plate and 500 μL culture medium containing chemotactic factors added to the bottom of the wells. Following culture in a CO_2 incubator for 24 hours, non-invasive cells and the matrigel matrix were removed. The cells on the lower surface of the membrane were then stained with trypan blue and counted under a microscope.

Tumorigenicity in nude mice

Tumorigenisity experiments were performed by injecting tumor cells in 0.1 mL PBS subcutaneously at BALB/c mouse axilla. Animal experiments were approved by the Committees on Animal Care and Use of Henan Provincial People's Hospital.To assess in vivo tumorigenicity, 10^3 to 10^6 cells were used for HeLa parental cells, or p16 gene silenced HeLa cells, respectively. Engraftment of tumors and subsequent growth was observed and monitored by vernier caliper. Tumor size was calculated using formula $V(mm^3)=ab^2/2$.

Statistical analysis

Data is expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) or unpaired Student's *t*-test was used to analyze the differences between groups. A two-tailed *p*-value of < 0.05 was considered statistically significant.

Results

p16 protein expression increased in HeLa tumor sphere cells and decreased in sh289 transfected HeLa cells

To identify the p16 gene expression level in HeLa sphere cells, HeLa cells were cultured in serum-free conditional medium and passaged four generations to compare p16 gene expression level in HeLa parental cells and tumor sphere cells. Only p16 protein, but not p14 protein was inhibited by the p16-sh289. The expression level of p16 protein was increased in tumor sphere cells. Also, Western blot analysis showed no significant difference in p16 protein level among passages (1 to 4) in the HeLa sphere cells (Figure 1).

Alternation of stem cell markers in HeLa cells transfected with p16-sh289

In the HeLa tumor sphere cells, the percentages of CD44+/CD24- and ABCG2+ increased significantly compared to that of the HeLa parental cells. In contrast, percentages of CD44+/CD24- and ABCG2+ decreased in HeLa cells transfected with the p16-sh289 lentivirus (Figure 2A, Table 1). The percentages of CD44+/CD24- and ABCG2+ were similar in HeLa parental cells and shRNA control cells.

Effects of p16 gene silencing on sphere formation in HeLa cells

Approximately 5×10^3 HeLa parental cells were seeded in six-well low adhesion culture plates and cultured in serum-free stem cell medium. In the HeLa parental cells, stem cell spheres were observed from day 3 and increased in size in the following days. In the HeLa cells transfected with the p16-sh289 lentivirus, no obvious sphere shaped cells were detected up to 14 days of culture in the same condition (Figure 2B).

Effect of p16 gene silencing on the invasive ability of HeLa cells

The HeLa cells were cultured in upper chamber in six-well plates with Transwell and incubated for more than 24

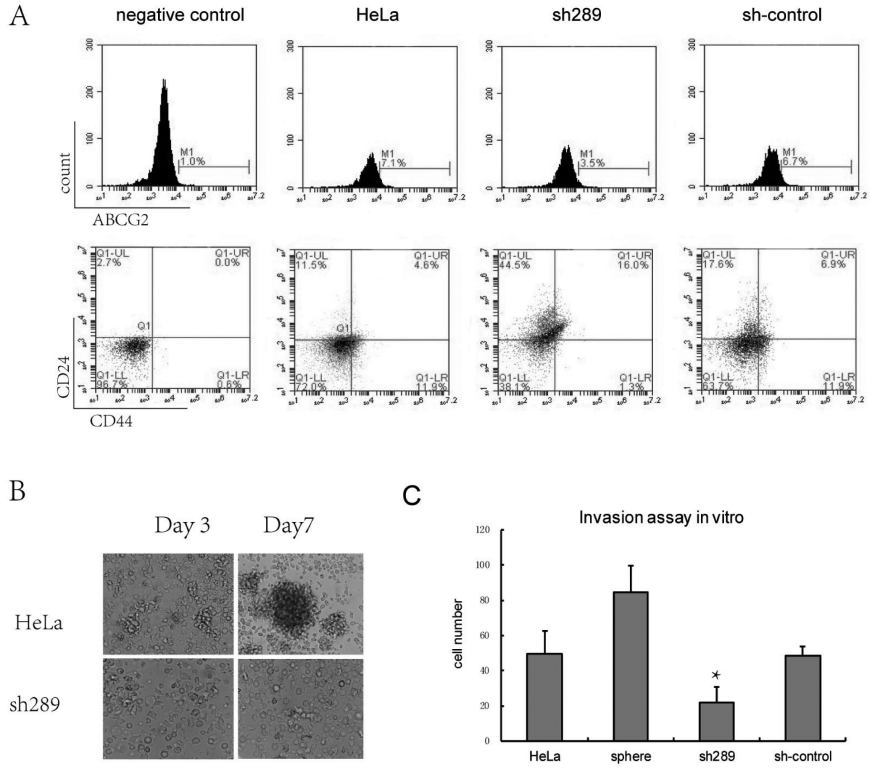

Figure 2. — Influence of p16 gene silencing on HeLa cell. A) Flow cytometry detection of ABCG2+ (upper panel) and CD44+/CD24- (lower panel) on HeLa cell surface. Negative control: cells incubated with fluorescence labeled homologous antibody; HeLa: HeLa parental cell line; sh289: HeLa cells with p16 gene silenced by sh289; sh-control: scrambled shRNA sequence as control. B) Sphere forming assay observed on day 3 and day 7. C) Cell number migrated through matrigel. *compared with that of HeLa parental cell line, $p < 0.01$.

Table 1. — *Relative percentage of CD44+/CD24-, ABCG2+ cells (%)*[$].

	CD44+/CD24-	ABCG2
HeLa parental	100±9.5	100±4.4
sh289 transfection	6.2±2.4*	21.0±3.7*
shRNA control	105.1±17.8	136.84±48.3
HeLa sphere	124.8±7.9#	318.3±30.1*

[$]Proportion of positive cells in each group compared to HeLa parental. Compared to HeLa parental cells, * $p < 0.01$; # $p < 0.05$.

Table 2. — *Tumor formation in vivo using HeLa cells with/without p16 gene silencing.*

Cell number	HeLa parental	sh289
1×10^6	4/4	5/5
1×10^5	5/5	4/5
1×10^4	3/5	1/5
1×10^3	0/5	0/4

Five mice were used in each group with different number of tumor cells.

hours. Among each control group (either no vector transfection or control shRNA transfection), there was no difference in the number of cells that passed through the matrigel. In the HeLa cells transfected with p16-sh289 however, the number of cells that passed through matrigel was significantly decreased (Figure 2C).

Tumor formation in mice

To establish the effect of p16 gene on tumorigenicity of HeLa cells in vivo, two groups of 5 BALB/c mice were injected with 10^6, 10^5, 10^4, and 10^3 cells, respectively. After six weeks, two mice died in total, one in each group. In HeLa parental cell group, 4/4, 5/5, 3/5, and 0/5 mice had formed tumors; while in p16 gene silenced HeLa cell group, 5/5, 4/5, 1/5, and 0/4 formed tumors (Table 2). Tumor size was measured using Vernier caliper and the volume was calculated using routine formula. Volume difference shown in Figure 3

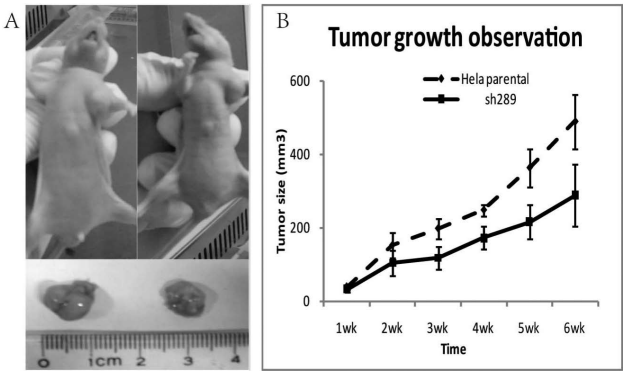

Figure 3. — Tumor formation in vivo. A) Upper figure showed tumor blocks in mice and lower figure showed tumor size at sixth week when sacrificed. Left: HeLa parental cell; Right: HeLa cells with p16 gene silencing. B) Tumor size observed and calculated. In p16 gene silencing group, tumor growth was retarded and there was a difference from week 2 compared to HeLa group ($p < 0.05$).

and the difference occurred from the second week after the tumor mass was visible. Mice were sacrificed, and tumors were excised and weighted in 10^6 groups at the end of the sixth week. Tumor weight was different between HeLa group (777.4 ± 110.4 mg) and p16 gene silenced HeLa group (437.1 ± 108.6 mg) ($p < 0.05$).

Discussion

p16 gene high expression in cervical tumor sphere cells

Previous studies have revealed that one of the characteristics of cancer stem cells is cell sphere formation in suspension condition when grown in serum free medium [12]. Similar results were reported in the cervical cancer cell lines CasKi and SiHa [13-15]. After grown in suspension at low density in serum-free sphere medium for approximately seven to ten days, the CasKi but not the SiHa cells produced tumor cell spheres in suspension in specific culture medium. The expressions level of Nanog, Nestin, OCT4, and EGFR were up-regulated in the 4th and the 7th passage but were then downregulated in the 10th and the 12th *in vitro* invasion. The detailed mechanism requires further investigation [14]. Four cervical cancer cell lines, HeLa, SiHa, CasKi, and C33A were compared for their sphere-formation abilities and the results showed that only HeLa and SiHa were able to form cancer cell spheres in another report [15]. The HeLa sphere formation cells expressed both E6 and E7. However, HeLa tumor sphere formation cells expressed 6.9-fold more E6 than the parent HeLa cells, which meant more important role of E6 played in cervical cancer stem cell. When HeLa sphere cells were treated with lentiviral-shRNA (18E6-1) targeting HPV E6, the sphere formation (sphere cell numbers) was significantly decreased, suggesting that E6 may have a profound impact on sphere formation cells self-renewal. Currently there is no report on whether p16 gene expression is high in tumor sphere cells or influences the characteristics of cervical cancer stem cells.

Recently, abnormal expression of p16 gene in cancer stem cells has been reported. Sphere forming cells derived from the pancreatic cancer cell line, Panc-1, showed increased resistance to gemcitabine, increased migration ability, and showed aberration of p16 gene expression [16]. The results of the current study show that sphere cells derived from parental HeLa cells have a higher p16 protein expression, indicating an important role for p16 gene in cervical cancer.

Silencing of the p16 gene may decrease the expression of stem cell markers in cervical cancer cells

Cancer stem cells are biologically distinct from other cancer cell types. They not only possess three hallmark features similar to stem cell-self renewal, extensive proliferation, and drive tumor formation, but also have high resistance to standard chemotherapy agents, high tumor expansion ability, and are difficult to treat with present therapies [17, 18].

ABCG2+ and CD44+ have been identified as markers of cancer stem cells in cervical cancer [12]. In the current study, the relationship between p16 gene activity and characteristics of cancer stem cell was investigated by using the cell markers, ABCG2, CD24, and CD44. The results of the present study indicate that in HeLa sphere cells, the cell number of CD44+/CD24- is increased compared with that of HeLa parental cells. However in HeLa cells transfected with p16-shRNA, the cell number of CD44+/CD24- is significantly decreased. The cell number of ABCG2+ showed a similar change. In the current study, the p16-shRNA silenced p16 gene expression but not p14. Taken together in cervical cancer, the downregulation of p16 gene expression reduces cancer stem cell number in the HeLa cell line. The results of the current study suggest a role for the p16 gene in reducing the occurrence, metastasis, and recurrence of cervical cancer.

Effects of p16 gene silencing on biological properties of HeLa cells

The *in vitro* invasion assay in the current study indicated that cells isolated from HeLa sphere formation cells showed the strongest penetration ability and that the number of cells invading through the matrigel was the highest. The number of cells that penetrated through the matrigel was less in the p16 gene silenced HeLa cell line than that of HeLa parental cell line. This data indicates that p16 is involved in the high invasion ability of cervical cancer cells and may regulate metastasis and recurrence of cervical cancer.

In addition, both HeLa parental cells and p16 gene silenced HeLa cells were cultured in cancer stem cell specific medium. At day 3, a number of cells were suspended and grew aggregately. These cells formed cell spheres containing multiple cells after a week. In p16 gene silenced HeLa cells however, formation of cell spheres was limited and the cells grew slowly in serum free medium. The results of the current study demonstrate that the p16 gene plays a significant role in maintaining cervical cancer stem cells. A recent report also showed p16 gene downregulation by siRNA in SiHa and HeLa cells inhibited cell proliferation, migration, and invasion [19].

Tumorigenic potential by p16 gene silencing HeLa cells decreased in vivo

Tumor sphere formation and invasion ability are feathers of tumor stem cell. However the more defined experiment is xenograft model for tumorigenic potential of tumor stem cells in vivo. In the present experiment, more p16 gene silenced cells were needed for tumor block formation in BALB/c mice. Tumor size increased faster in HeLa parental cell group than that in p16 gene silencing group. Tumor weight after six weeks was heavier in HeLa group than that in gene silencing group when 10^6 cells were injected. These data suggested that p16 gene was important for tumor initiation and expanding of HeLa stem cell.

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