VPS50 is required for the proliferation and survival of cervical cancer cells

Huiya Zhang1*,1†, Pingping Tao2*,1, Jiangjing Shan1, Yanmin Zhou1, Yungen Wang1, Yuhong Xu1,2

1 Department of Gynecology, Shaoxing People’s Hospital (Shaoxing Hospital, Zhejiang University School of Medicine), 312000 Shaoxing, Zhejiang, China
2 Department of Obstetrics and Gynecology, Shanghai Pudong New Area People’s Hospital Affiliated to Shanghai Health University, 201299 Shanghai, China

*Correspondence: zhanghuiya1983@126.com (Huiya Zhang); yuhongxut988@sina.com (Yuhong Xu)
† These authors contributed equally.

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Objective: Study of important molecules involved in the pathogenesis of cervical cancer will be of great significance for the early screening and treatment. Growing evidence suggests that Vacular protein sorting (VPS) proteins play roles in cancer biology. However, the role of VPS50 in cancers has not been explored. The purpose of this study is to explore the role of VPS50 in the progression of cervical cancer.

Methods: In silico analysis was performed to explore the expression and mutation of VPS50 in several cancers. A shRNA against VPS50 were constructed to knockdown VPS50 in SiHa cells, which is a cervical cancer cell line. Cell growth was detected by clone formation assay and Celigo cell counting assay. Apoptosis rate was measured by flow cytometry and Caspase-3/7 Assay. PathScan intracellular signaling arrays kit was used to detect the changes of signaling molecules involved in Stress and Apoptosis pathway after VPS50 knockdown in SiHa cells. Results: VPS50 is highly expressed in various cancers including cervical cancer, and various VPS50 mutations exist in cancer cells. Down-regulation of VPS50 expression results in decreased cell proliferation and an inhibition of colony formation of SiHa cells. Moreover, VPS50 knockdown could significantly induce the apoptosis of SiHa cells by activating the Stress and Apoptosis Pathway.

Discussion: Our study has demonstrated that VPS50 plays an essential role in the progression of cervical cancer and may serve as a potential therapeutic target for cervical cancer.

Keywords
Cervical cancer; EARP; VPS50; Proliferation; Apoptosis

1. Introduction
Cervical cancer (CC) is one of the most common gynecologic tumors and is responsible for more than 300,000 deaths worldwide each year [1, 2]. In developed countries, early detection through screening has led to a substantial decline in mortality from CC. However, mortality remains high in developing countries and in low-income families [2, 3]. Most patients diagnosed with CC receive radiotherapy or chemotherapy, resulting in a heavy financial burden [2, 4]. Furthermore, the clinical outcome varies greatly and some patients diagnosed with early stage disease later develop tumor metastasis resulting in shortened survival [5]. The molecular mechanisms that underlie the progression of CC remain unclear, thus hindering the development of more effective treatments. Hence, the identification of important pathways and molecules involved in the pathogenesis of CC will be of great significance for early detection through screening and for improved treatment.

Cancer cells are able to carry out relatively minor modifications to normal physiological processes, which can in turn result in markedly altered phenotypes. Endocytosis has been identified as one such physiological process that undergoes modification during carcinogenesis [6, 7]. Many cancer cell types lose the ability to regulate the function of important growth control receptors such as epidermal growth factor receptor (EGFR) and ErbB family of receptor tyrosine kinases (RTK). These key regulators of growth cannot be internalized, recycled or degraded by the cancer cell, thereby accelerating tumor development [6–8]. The process of unregulated receptor endocytosis provides a range of potential targets for the treatment of cancer. In theory, the removal or inhibition of oncogenic proteins that are defective for endocytosis might inhibit cancer progression. Significant advances have recently been made in understanding the molecular changes that occur to the endocytic pathway during cancer progression [7, 8]. A multi-subunit tethering complex called endosomal-associated recovery protein (EARP) is involved in endocytosis. This complex consists of four vacuolar protein sorting-associated (VPS) proteins that are referred to as VPS50, VPS51, VPS52 and VPS53. There is growing evidence to suggest that VPS proteins play important roles in tumor biology. VPS52 can induce apoptosis in gastric cancer and suppress the growth of this cancer type [9]. VPS53 may have multiple roles in a variety of human tumor types. For example, in human CC it can act as a tumor suppressor by regulating the apoptosis pathway [10, 11]. VPS50, also known as CCDC132 or Syndetin, is another component of EARP. To date, a role for VPS50 in tumors has yet to be reported [8]. In the present study we found that VPS50 is highly expressed in various cancer types, including human
CC. Knockdown of VPS50 expression inhibited the proliferation of CC cells, enhanced their apoptosis and prevented colony formation. Furthermore, molecules involved in the Stress and Apoptosis-related signaling pathway were significantly up regulated according to the Path Scan intracellular signaling array. Therefore, these results suggest that VPS50 is required for the growth of CC cells.

2. Methods

2.1 Cell culture

The SiHa human CC cell line was purchased from the Chinese Academy of Sciences (Shanghai, China) and grown in ATCC-formulated Eagle’s Minimum Essential Medium (Catalog No. 30-2003) containing 10% fetal bovine serum ( Gibco, Scoresby, Australia) and 100 U/mL penicillin/streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO₂ chamber.

2.2 In silico analysis

The cBioPortal database (http://www.cbioportal.org/) was used to extract expression and mutation datasets for VPS50 in various cancer cell lineages including adrenocortical carcinoma, bladder urothelial carcinoma, invasive breast carcinoma, CC, glioblastoma, lung adenocarcinoma, ovarian serous cystadenocarcinoma and stomach adenocarcinoma.

2.3 q-PCR

Total RNA was prepared using the RNeasy Mini kit (QIAGEN, Hilden, Germany) as described by the manufacturer. It was then reverse-transcribed using the SuperScript II First-Strand Synthesis System (Invitrogen). qPCR analyses were performed using SYBR green detection (ABI, Auckland, New Zealand) and the following primers (GeneChem Co., Shanghai, China): VPS50 forward, 5’CATCTGGGGATACGCTGTATG3' and reverse, 5’GTAGTTCATTGCCGTTGA G3'; GAPDH forward, 5’GTAGTTCACTGGCGACGCTGTATG3' and reverse, 5’GTAGTTCACTGGCGACGCTGTATG3' and reverse, 5’CACCCTGTGCTGTAGC-CAA3'.

2.4 Western blot

Western blotting was performed as described previously [12]. Briefly, SiHa cells were harvested and lysed with RIPA buffer (10 mM Tris-HCL, pH 7.6, 1 mM ethylenediamine tetra-acetate (EGTA) and 1,2, bis,2 amino-ethylenethane-NNNN-tetra-acetic acid (EDTA), 0.1% SDS, 1% Triton X-100, 0.1% NA-Deoxycholate, 1× protease inhibitor cocktail and 1mM Phenylmethylsulfonyl fluoride (PMSF)) containing protease inhibitor. Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Protein (20 µg) was separated using dodecyl sulfate, sodium salt (SDS)-Polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes and incubated with primary antibodies: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Signals were visualized using enhanced chemiluminescence following incubation with the appropriate secondary antibodies (anti-VPS50, Cat# HPA026679, Sigma; anti-GAPDH, Cat# sc-32233, Santa-Cruz).

2.5 Colony formation assay

The colony formation assay was performed as described previously [13]. SiHa cells were seeded into 6-well plates at 200 cells/well with 2 mL of medium and grown at 37 °C for 2 weeks. Cells were then fixed in 4% Paraformaldehyde, stained with 0.1% Crystal Violet (Cat#C8470, Solarbio, Beijing, China) and the total number of colonies per well was counted. Three independent replicates were counted.

2.6 Construction of the shRNA lentiviral vector and transfection

Lentiviruses expressing short hairpin RNAs (shRNAs) that target the VPS50 gene sequence (5’TCAAGAAAGCCTCAGT GAT3’) and scramble shRNA as the control were obtained from Shanghai GeneChem Co., Ltd (Shanghai, China). The corresponding shRNAs were cloned into a pGC-SILgreen fluorescent protein lentiviral vector to generate recombinant lentiviral shRNA expression vectors. For the packaging of lentivirus, HEK293FT cells were co-transfected with shRNA plasmid constructs and with packaging plasmids using Fugene HD transfection reagent as recommended by the manufacturer. Lentivirus particles were purified by ultracentrifugation and endpoint dilution assay was used to determine their titer. SiHa cells seeded into 6well plates (3 × 10⁴ cells/well) were infected with either shVPS50 lentivirus or shCtrl-lentivirus.

2.7 Cell proliferation assay using the Celigo Image Cytometer

SiHa cells from logarithmic growth phase were resuspended in culture medium and seeded into 96-well plates at a density of 1,000 cells/well. Starting the next day, a Celigo Image Cytometer (Nexcelom Bioscience, Lawrence, MA, USA) was used to measure at 488 nm wavelength each day for five consecutive days, thus generating cell growth curves.

2.8 Apoptosis assay

The annexin V-allophycocyanin (APC) apoptosis detection kit (Cat# 88-8007, Ebioscience, Shanghai, China) was used to assess cell apoptosis as recommended by the manufacturer. Cells treated with shVPS50 or shCtrl were resuspended at a density of 1 × 10⁶ cells/mL. One million cells were stained with annexin V-APC (10 µL) for 15 minutes at room temperature, followed by flow cytometric analysis (Guava easy Cyte HT, Millipore, Burlington, MA, USA). For the Caspase 3/7 activity assay, SiHa cells were seeded into 96-well plates (1000 cells/well) and grown for one day. Caspase 3/7 activity was then detected using the Caspase Glo® 3/7 Assay (Cat# G8091, Promega Corporation, Madison, WI, USA) according to manufacturer instructions.

2.9 Intracellular signaling array

Cells were harvested and then lysed on ice with 100 µL of lysis buffer for 5 min. The lysates were centrifuged at 10,000 g for 10 min at 4 °C. The PathScan® Stress and Apoptosis Signaling Antibody Array Kit (Fluorescent Readout, Cat# 12923, Cell Signaling Technology, Danvers, MA, USA) was
used to detect intracellular signaling molecules as per the manufacturer’s instructions. Fluorescent images of the slide were captured with the Odyssey® Infrared Imaging System (LI-COR, Lincoln, NE, USA) and spot intensities were quantified using Image Studio analysis software.

2.10 Statistical analysis

Data are shown as the mean ± SD. Two-tailed t-test was used to compare results for two groups. A p-value of <0.05 was considered to show statistical significance. Experiments were independently repeated at least three times.

3. Results

3.1 VPS50 Expression in cancer

To examine the role of VPS50 in oncogenesis, VPS50 expression and mutation in various cancer types was investigated using published datasets. Data from the cBioPortal database (http://www.cbioportal.org/) showed high VPS50 expression in adrenocortical carcinoma, bladder urothelial carcinoma, invasive breast carcinoma, CC, glioblastoma, lung adenocarcinoma and ovarian serous cystadenocarcinoma, but low expression in gastric adenocarcinoma (Fig. 1A). Various genetic alterations to VPS50 were also found in these cancer types, including gene amplification, point mutation and fusion, (Fig. 1B), with the former being the most frequent. The highest mutation frequency for VPS50 was found in gastric cancer, followed by lung cancer. Approximately 2% of all VPS50 mutations occurred in CC. The above results indicate that VPS50 may play a role in various cancer types. To investigate a potential role for VPS50 in CC, qPCR was carried out on two CC cell lines, Hela and SiHa. This confirmed that VPS50 was expressed at a high level in CC (Fig. 1C).

3.2 Lentivirus-mediated RNAi suppresses VPS50 expression in SiHa cells

To examine the function of VPS50 in CC, SiHa cells were infected with lentivirus that expressed shRNA directed against VPS50 (shVPS50), or with lentivirus that expressed a non-target control shRNA (shCtrl). This generated cells lines with stably repressed VPS50, and control cells with normally expressed VPS50, respectively. qRT-PCR assays showed the VPS50 mRNA level was significantly reduced by 50% in shVPS50-treated SiHa cells compared to shCtrl-treated SiHa cells (Fig. 2A). Moreover, VPS50 protein levels were also reduced in shVPS50 SiHa cells (Fig. 2B). These results indicated that lentivirus-mediated shRNA silencing efficiently inhibited the expression of endogenous VPS50 in SiHa cells at both the RNA and protein levels.
Fig. 3. Colony formation of VPS50 knockdown and control SiHa cells. (A) Representative images of colony formation of VPS50 knockdown and control SiHa cells. (B) Quantification of the total number of colonies in VPS50 knockdown and control SiHa cells. Error bars represent ± SD; n = 3, **p < 0.01, 2-tailed t-test.

Fig. 4. Effect of VPS50 knockdown on the proliferation of SiHa cells. (A) Representative images of SiHa cells infected with either shCtrl or shVPS50 at different time points. (B,C) Proliferation data are shown as cell numbers (B) and fold-changes of cell numbers (C). Triple experiments were performed. Values were represented as mean ± SD. n = 3, **p < 0.01, 2-tailed t-test.
3.3 VPS50 knockdown inhibits colony formation of SiHa cells

Colony formation is an anchorage-independent growth assay considered to be the most stringent assay for detecting cell survival and proliferation in vivo. It was therefore used to determine the colony-forming ability of SiHa cells following VPS50 knockdown. Both the number and size of colonies were reduced in the VPS50 knockdown cells compared to the control cells (Figs. 3A, 4B), indicating suppression of colony formation in SiHa cells following VPS50 knockdown. This may be due to the poor viability and decreased proliferation of SiHa cells following VPS50 knockdown.

3.4 VPS50 knockdown reduces the proliferation of SiHa cells

Reduced cell proliferation is a possible factor leading to decreased clonogenic capacity. To investigate the role of VPS50 in proliferation, cells were seeded at 2 × 10^4 cells/well into 96-well plates and counted for 5 consecutive days using the Celigo Image Cytometer. Significant inhibition of cell proliferation was observed after 3 days in the VPS50 knockdown SiHa cells (Fig. 4A). Knockdown of VPS50 significantly inhibited the growth of SiHa cells (Fig. 4B, C), demonstrating that VPS50 is an important factor for SiHa cell proliferation.

3.5 VPS50 knockdown in SiHa cells activates apoptosis

Increased apoptosis may account for the observed decrease in clone formation. Hence, cell death was studied in VPS50 knockdown cells by evaluating annexin V-APC positive cells in the shVPS50-treated group compared to the control group (15 ± 0.48% vs. 3.6 ± 0.12%, p < 0.01) (Fig. 5A, B). Similarly, Caspase3/7 activity was significantly higher in shVPS50-treated cells compared to shCtrl-treated cells (46,292 ± 375 vs. 7,826 ± 207, p < 0.01) (Fig. 5C). These results indicate that VPS50 downregulation promotes apoptosis in SiHa cells.

3.6 VPS50 knockdown induces Stress and Apoptosis pathway activation

A PathScan Stress and Apoptosis Signaling Antibody Array Kit was used to investigate possible molecular mechanisms by which VPS50 affects the survival of SiHa cells. VPS50 knockdown was associated with increased expression of cleaved PARP, cleaved Caspase-3, cleaved Caspase-7, IkBa, Survivin and a-Tubulin (Fig. 6). Furthermore, significantly increased phosphorylation levels for components of the stress and apoptosis pathway were detected in shVPS50-treated SiHa cells. These included p-ERK1/2, p-Bad, p-HSP27, p-p53, p-p38 MAPK, p-Chk1, p-Chk2 and p-eIF2 (Fig. 6). These results suggest that VPS50 downregulation induces apoptosis of SiHa cells by activating the Stress and Apoptosis pathway. Collectively, the increased apoptosis and decreased cell proliferation caused by VPS50 knockdown is likely to decrease the tumor forming ability of CC cells.

4. Discussion

A better understanding of the molecular mechanisms that underlie the progression of CC could lead to development of more effective therapies for this disease. Recently, many studies have implicated endocytosis in the development and progression of cancer. Further research into this process may therefore uncover potential targets for the treatment of CC [6, 7]. Endosomal associated restorer protein (EARP) is a vital component of the endocytic pathway. Two of its subunits, VPS52 and VPS53, have already been identified as having important roles in tumor development [8, 14–16]. However, the role of the most recently identified ERAP subunit, VPS50, remains unclear and only a few studies have so far been reported. VPS50 is expressed at high levels in T cells from atopic dermatitis and may be involved in the aetiology of this disease [14]. VPS50 is also expressed in neurons, where it may regulate vesicle acidification and therefore have a role in synaptic function. Functional mutations in VPS50 may also contribute to neurodevelopmental disorders [15, 16]. Cancerous pancreatic ductal fluid contained increased levels of VPS50 compared to normal pancreatic ductal fluid, suggesting it may also play a role in this cancer type [17]. By analyzing public data, the present study found high levels of VPS50 expression in adenocortical carcinoma, bladder urothelial carcinoma, invasive breast carcinoma, CC, glioblastoma, lung adenocarcinoma and ovarian serous cystadenocarcinoma, but low levels in gastric adenocarcinoma. It is worth noting that various genetic alterations to VPS50 have been reported in these tumor types, including gene amplification, point mutation and fusion. Amongst these, gene amplification is the most frequent alteration. Together, these findings suggest VPS50 may have an important role in tu-
morigenesis. Whether VPS50 expression or genetic alteration is associated with the clinical outcome of cancer patients requires further investigation.

The present results revealed a potential role for VPS50 in CC. Using lentivirus-mediated shRNA knockdown, it was demonstrated here that VPS50 is required for CC cell growth. Knockdown of VPS50 expression led to inhibition of CC cell proliferation, enhanced apoptosis of CC cells, and prevention of colony formation. In contrast to the apparent oncogenic effects of VPS50, two other members of the ERAP complex (VPS53 and VPS52) are thought to exert inhibitory effects on the growth of cancer cells. VPS52 induces apoptosis and acts as a suppressor of gastric cancer cells [9]. VPS53 has been shown to suppress tumor growth in human CC by regulating apoptosis pathways [10, 11]. The possible reasons for these apparent functional differences between VPS proteins require further investigation, but may be due to the different cellular contexts in which they were studied. The different subunits could participate in the formation of alternate complexes depending on the cellular context.

The Path Scan Stress and Apoptosis Signaling Antibody Array was used here to investigate the molecular mechanisms by which VPS50 can affect CC cell growth. The results suggest that a large set of molecules involved in stress- and apoptosis-related signaling were significantly altered by VPS50 knockdown, including cleaved Caspase-3/7, Survivin, p-ERK1/2, p-Bad, p-p53, p-p38 MAPK and p-eIF2. Cleaved PARP-1 and cleaved Caspase-3/7 are both well-known apoptotic markers. Activation of p-p38 MAPK and p-p53 triggers programmed cell death [18–20]. In contrast, up-regulation of Survivin, p-Chk1/2, p-ERK and p-eIF2α inhibits apoptosis [21–23] in what could be a secondary reaction caused by self-rescue of the apoptotic cells. Activation of these stress- and apoptosis-related molecules following VPS50 knockdown strongly suggests that VPS50 is necessary for the survival of CC cells. However, a full understanding of the molecular mechanism involved requires further study.

5. Conclusions
This is the first report of high VPS50 expression in CC and the first to suggest this endocytosis-related protein has an important role in CC pathogenesis. Downregulation of VPS50 expression in SiHa cells resulted in the inhibition of colony formation due to decreased cell proliferation and increased cell death. The mechanism by which this occurs is through activation of the Stress and Apoptosis pathway, thus preventing the survival of SiHa cells. These results provide strong evidence that VPS50 could serve as a potential therapeutic target for CC.

Author contributions
ZHY and TPP designed the research study. XYH and SJJ performed the research. XYH and WYG provided help and advice on the lentivirus infection experiments. SJJ and ZYM provided help and suggestions for apoptosis experiments. ZYM and WYG analyzed the data. ZHY and TPP wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate
Not applicable.

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Conflict of interest
The authors declare no conflict of interest.

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