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Original Article

Abnormal activation of notch 1 signaling causes apoptosis resistance in cervical cancer

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Abstract: Notch1 signaling pathway is an evolutionarily conserved and crucial regulator to determine cell fate and differentiation. Notch1 is often over expressed in several cancers, which plays an essential for cancer cell proliferation, survival, invasion and metastasis. The oncogenic function of Notch1 signaling in cervical cancer progression is not well-characterized. In the present study, we showed that Notch1 is significantly enhanced in cervical cancer tissues. Similarly, the relative mRNA and expression of Notch1 protein are significantly upregulated in cervical cancer cell lines such as HeLa and SiHa. Further, we have performed RNAi for *NOTCH1* depletion to determine its specific role in cervical cancer progression. Flow cytometry analysis revealed that *NOTCH1* depletion leads to activation of apoptotic cell death in cervical cancer. Further, the *NOTCH1* depleted cells showed increased sensitivity towards DNA-targeting drugs and therefore cell viability was reduced efficiently. Altogether, our findings suggest that Notch1 overexpression in cervical cancer cells was involved in tumorigenesis and apoptosis resistance of cervical cancer.

Keywords: Apoptosis, cell proliferation, cervical cancer, chemoresistance, Notch1 signaling

Introduction

Globally, cervical cancer is a serious health concern and one of the foremost causes for cancer mortality among women [1]. Persistence of human papillomaviruses (HPVs) are the essential risk component for the cervical cancer progression. HPVs are a small group of heterogenous, non-enveloped virus possessing circular double stranded DNA which infects the epithelial cells of skin and mucosa. Among HPVs, HPV-16 and 18 were shown to be high risk, which accounts for more than 70% of cervical cancer worldwide [2]. Particularly, HPV-18 contributed to more than 12% of squamous cell carcinoma (SCC) and approximately 40% of cervical adenocarcinoma (ADC) [3]. Despite recent advances in the early diagnosis and different treatment methods (includeing chemotherapy, Immunotherapy and radiotherapy), the outcome of treatment is still poor and hence the mortality rate increases. The underlying cause for treatment failure and tumor regeneration is due to the presence of cancer stem cells (CSC) which have stemness properties and overexpression of ATPase-binding cassette transporter proteins (ABC proteins). These ultimately contribute to apoptosis resistance/multi-drug resistance of cancer cells. As a result, when chemotherapy is withdrawn there is a persistence of a small subset of CSCs, causing cancer relapse. Therefore, tumor recurrence can be efficiently accomplished by increased cell proliferation, tumor progression, and metastasis [4-6]. Elucidation of an underlying mechanism of apoptosis resistance and cervical cancer carcinogenesis is needed. It has been well documented in recent studies that disruption in the regulation of cell signaling pathways is significantly associated with the cancer development [7, 8]. Accordingly, deregulation of COX2, NF-κB, p53, Wnt/β-catenin, and Notch are suggested as crucial factors, which are corelated with cervical cancer progression [9, 10]. Hence, determination and exploration of genetics and mechanisms of such signaling pathways and its downstream targets are crucial for the innovation of precise therapeutic drugs to achieve better treatment regimen.

Notch signaling is an evolutionarily highly conserved pathway that plays a critical role in regulating cell destiny, proliferation, survival, and differentiation. In addition, Notch1 protects the

cells from apoptosis and various apoptotic stimuli [1, 11-15]. Notch receptors belong to the class of large, transmembrane proteins comprising epidermal growth factor-like repeats with an N-terminal extracellular domain for ligand binding and C-terminal cytoplasmic domain for signal transduction [16]. So far, four Notch receptors [1-4] were identified in mammals that are activated by cell surface ligands such as Serrate (Jagged) and Delta (Delta-like) ligands. Upon activation, these Notch receptors are subjected to proteolytic cleavage by enzymes such as disintegrin, metalloproteinase enzyme, and γ-secretase, causing release of NCID (Notch intracellular domain) from the membrane [17]. Consequently, the released NCID is transported to the nucleus and activates the transcriptional factors and downstream-targeted genes [18]. Among the four Notch receptors, NOTCH1 was dominantly expressed in different cancer tissues [19].

Notch1 signaling acts either as a tumor suppressors or as oncogene in several cancers [20]. Reports have been demonstrated that Notch1 signaling was aberrantly expressed in different types of cancers (colon, breast, liver, cervical, thyroid) and contributed to cancer progression [1, 20-25]. In addition, reports suggesting that Notch1 is essential for cancer cell growth, proliferation, survival, apoptosis, and maintenance of cancer stem cells [26]. So far, few studies have been reported about the involvement of Notch1 (and its ligand) in cervical cancer progression [1, 9]. However, the understanding of precise role of Notch1 and its molecular events as a prognostic marker in cervical cancer progression is still lacking. Therefore, in the present study, we have analysed the expression pattern of Notch1. Its involvement specifically in apoptosis was evaluated by RNAi-mediated knockdown in cervical cancer cells. Our data revealed that Notch1 is involved in cervical cancer progression and apoptosis resistance and these findings should provide insight for future detailed research into the mechanistic events of Notch1-mediated cervical cancer cell survival.

Materials and methods

Cancer sample collection

Cervical cancer biopsies (n=30) and the corresponding normal tissues (n=30) were obtained from the patients during surgery at Department of Obstetrics and Gynaecology, The People's Hospital of China, Three Gorges University. We have followed ethical rule approved by the Three Gorges University Ethical committee and the tissues were obtained in accordance with oral consent from the patients. The following details were included in the sample collections: tumor grade-High grade; stage-moderate (5) well differentiated (25); origin-squamous cell carcinoma (WDSCC). The obtained control and cancerous tissues were subjected to histopathologic examination followed by haematoxylin-eosin staining.

Culturing of cell lines and cervical cancer tissues

PBS solution (with antibiotics) was used to wash the cancer tissues and they were incubated overnight in DMEM/F12 (Invitrogen) with Penicillin and streptomycin. Subsequently, enzymatic digestion was performed for 1 h in PBS solution supplemented with 1 mg/mL of collagenase and 15 μg/mL of hyaluronidase. The prepared cell monolayers were cultured and serially passaged in cell culture dishes (Fishers Scientific) with DMEM with 10% FBS with antibiotics at 37°C with 5% CO_2 supply.

Similarly, cell lines such as HeLa, SiHa, and human embryonic kidney cells (HEK) were purchased from Thermo Fisher. All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (FBS; Invitrogen Life Technology), incubated at 37°C in a humidified 5% CO_2 & 95% air atmosphere.

RT-PCR analysis

For the cellular RNA extraction, RNA extraction kit from Thermo Fisher and its guidance protocol was used. The RT-PCR consensus primers were used as previously described: *NOTCH1*: F-CTGGTCAGGGAAATCGTG and R-TGGGCAGTGGCAGATGTAG [27]; *GAPDH*: F-ATGTCGTGGAGTCTACTGGC and R-TGACCTTGCCCACAGCCTTG [28]. RT-PCR cycle employed was: Denaturation at 95°C for 10 s (50 cycles); annealing at 54°C for 15 s; extension at 70°C for 15 s and the final extension at 72°C for 10 min. All the amplicons were visualized by 1.2% agrose gel and quantification graph was made with the level of

NOTCH1 mRNA expression which was adjusted with *GAPDH* (used as control).

RNA interference

Approximately 1 × 10^6 cells/well (6-well plate) were cultured for 24 hours at 37°C and subsequently RNAi transfection was executed with the aid of transfection reagent Lipofectamine®2000 (Invitrogen) as described in the protocol. The *NOTCH1* RNAi sequences were as mentioned previously [1]. si*NOTCH1*-5'-GATCCTGGCGGGAAGTGTGAAGCGT-3' (Gene Pharma Co., Ltd).

Flow cytometry analysis

We used propidium iodide staining (PI) kit (Sigma-Aldrich) to evaluate the rate of apoptosis by flow cytometry. The post-transfection RNAi cells were fixed in 70% ice-cold methanol, followed by overnight incubation with PI and RNase in dark condition at 37°C. Finally, cells were analyzed by flow cytometry and the signal values obtained were represented as a graph that indicates percentage of cells undergoing apoptosis.

Cell proliferation assay

10^6 cells were seeded/well in a six-well plate. The rate of cell proliferation was measured consecutive days for 7 days. Cells were treated with CCK-8 (10 µL) solution for 2-3 hours and subjected to measurement of growth rate at 450 nm. These values obtained were used to construct the growth curve.

Chemoresistance assay

Approximately 10^6 cells were seeded in a 96-well plate and overnight incubation was given. Subsequently, cells were treated with 5-fluorouracil (5-FU) 10 µg/mL; Cisplatin (20 µmol/L), Paclitaxel (2 µmol/L) and further incubated for 48 hours. Cell resistance was estimated by: Cell resistance rate (%) = (experimental group OD_{450} value/control group OD_{450} value) × 100, as previously performed [29].

Western blot analysis

Cell lysates were prepared by using lysis buffer (SDS; Sigma-Aldrich) and the proteins were separated on 10% SDS-PAGE and PVDF membranes was used to transfer the proteins (Millipore). After blocking, membranes were subjected to primary antibody incubation with anti-human Notch1 (Polyclonal-goat; 1:400; from Santa Cruz) and anti-human GAPDH (monoclonal-mouse; 1:5000; from Cell Signaling) antibodies overnight at 4°C. Subsequently, incubation with horseradish peroxidase-conjugated (rabbit anti-goat; dilution: 1:5000 from Thermo Fisher for 1 h and the protein signal was visualized by enhanced chemiluminescence (ECL) kit (Biorad). For the blot quantification, the expression of Notch1 was adjusted by GAPDH signal (loading control).

Statistical analysis

Student's *t*-test was employed for the comparison between two groups and SPSS software of version 16.0 was used for further statistical analysis. We considered the difference significant when *P* values were *, <0.05 and **, 0.01.

Results

Enhanced expression of Notch 1 in cervical cancer

We started our investigation with human cervical cancer tissues and the corresponding control tissues for the evaluation of endogenous expression of Notch1. By western blot analysis, we found significantly enhanced expression of Notch1 protein in cervical cancerous tissues when compared to the controls (**Figure 1A**). However, a few cancer samples showed a moderate level of Notch1 expression. Further, quantification of the mean intensity of Notch 1 from western blots confirmed the significantly elevated (P<0.01) expression of Notch 1 in cervical cancer tissues (**Figure 1B**). We also observed that 90% (27/30) of the cervical cancer tissues showed positivity of Notch1 expression whereas only 10% (3/30) of the control tissues were Notch1 positive. Subsequently, we performed the same type of investigations in cervical cancer cell lines such as HeLa and SiHa. Our RT-PCR analysis revealed that relative mRNA expression for Notch1 is highly upregulated (3.5 fold increase) in HeLa as well as SiHa cells, compared to HEK cells, used as a control (**Figure 2A**). Again, Notch1 protein expression was significantly greater in HeLa and SiHa (P<0.01) than control cells (**Figure 2B** and **2C**). Therefore, these findings suggest that Notch1 is prone to hyperactivation in both cervical can-

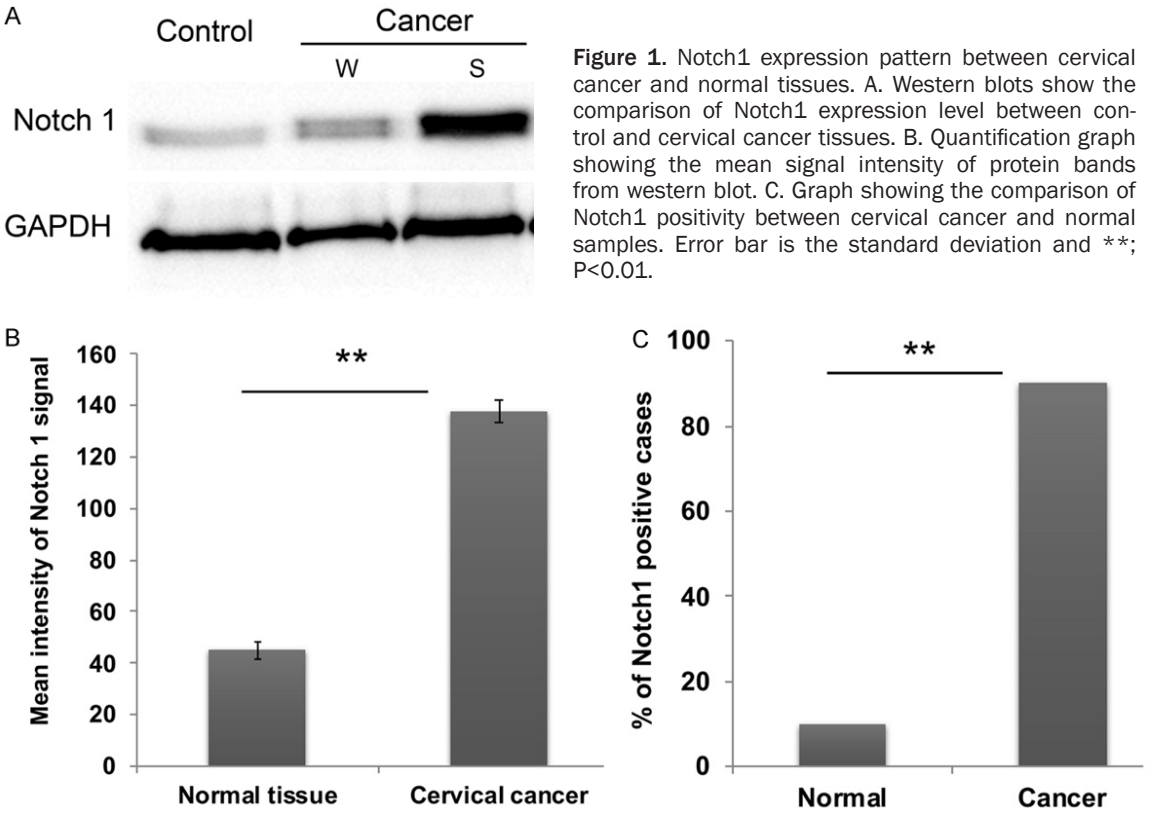

Figure 1. Notch1 expression pattern between cervical cancer and normal tissues. A. Western blots show the comparison of Notch1 expression level between control and cervical cancer tissues. B. Quantification graph showing the mean signal intensity of protein bands from western blot. C. Graph showing the comparison of Notch1 positivity between cervical cancer and normal samples. Error bar is the standard deviation and **; P<0.01.

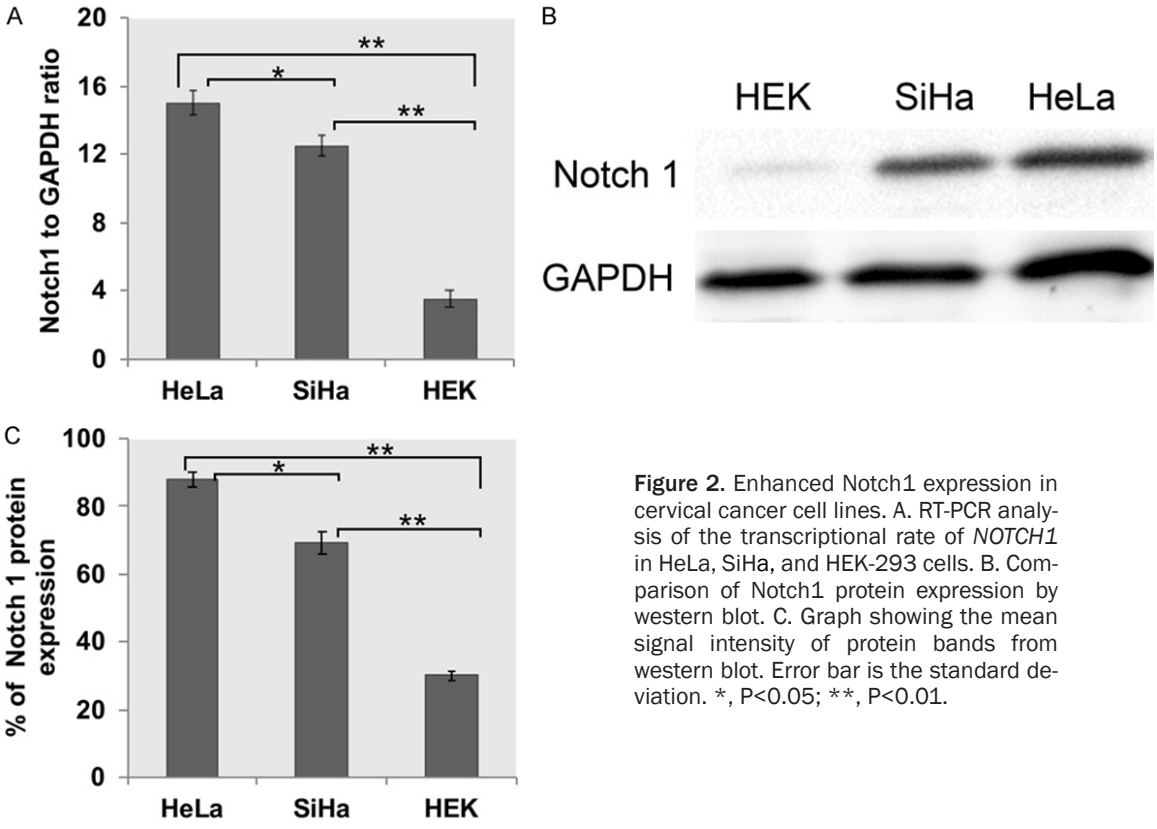

Figure 2. Enhanced Notch1 expression in cervical cancer cell lines. A. RT-PCR analysis of the transcriptional rate of *NOTCH1* in HeLa, SiHa, and HEK-293 cells. B. Comparison of Notch1 protein expression by western blot. C. Graph showing the mean signal intensity of protein bands from western blot. Error bar is the standard deviation. *, P<0.05; **, P<0.01.

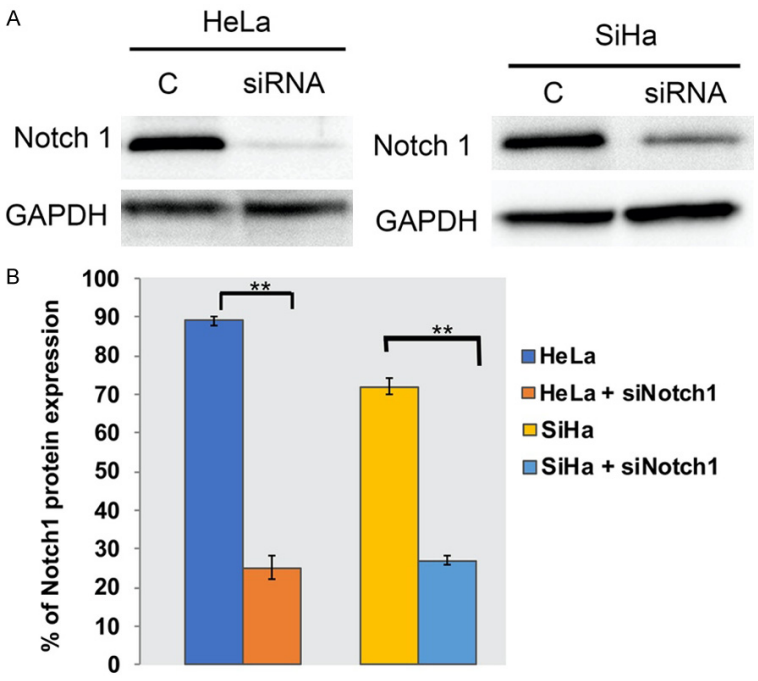

Figure 3. Depletion of *NOTCH1* expression by RNAi. A. Western blot showing significantly reduced Notch1 expression in HeLa and SiHa cells, that were transfected with *NOTCH1* RNAi. B. Quantification graph showing the mean signal intensity of protein bands from western blot. Error bar: standard deviation and **; P<0.01.

cer tissues and cell lines, which might be associated with cervical cancer progression.

Effect of Notch 1 depletion on apoptosis induction and cell proliferation

We knocked down *NOTCH1* by RNAi in order to determine its function in cervical cancer progression. Notch1 expression was significantly (P<0.01) downregulated in HeLa and SiHa cells, confirmed by western blotting (**Figure 3A** and **3B**). Next, we performed flow cytometry-mediated quantitative analysis for apoptosis induction of the control and Notch1 depleted cells. There was a significantly enhanced rate of apoptosis induction (P<0.01) in HeLa and SiHa cells where Notch1 was depleted, versus the corresponding controls (**Figure 4A**). Data suggest that apoptosis was initiated in *NOTCH1* RNAi cells and therefore we further performed a chemoresistance assay against 5-FU, cisplatin, and paclitaxel for the cell viability evaluation. Upon treatment with the above chemotherapeutic drugs, *NOTCH1* depleted cells had increased sensitivity towards the drugs and viability declined significantly more than controls (**Figure 4B** and **4C**). Finally, our *in vitro* cell proliferation data showed that proliferation/growth rate was efficiently impeded in Notch 1 RNAi cells (**Figure 5**). Altogether, our data suggest that *NOTCH1* downregulation makes cancer cells more susceptible towards DNA targeting drugs which lead to apoptosis induction and cell death. Furthermore, the inhibited cell growth curve suggests a prominent role of Notch1 in cancer cell proliferation.

Discussion

The persistence of high-risk Human Papillomavirus (HPV) infection, such as HPV-16 and HPV-18, is inextricably linked with cervical cancer growth [30]. In worldwide, cervical cancer is a major health concern, the third most common cancer and fourth most frequent cause for cancer related death among women. Incidence and mortality remain high despite diagnosis and therapeutic drugs [31]. Evidence suggests that abnormal activation or downregulation of signaling pathways and their downstream targets leads to deregulated cell proliferation and apoptosis inhibition which are required for malignant transformation [1, 32]. One such pathway, Notch1 signaling, was often hyperactivated in different cancers. Notch1 signaling is an evolutionarily conserved pathway that transmits signals from the cell surface to nucleus, whose essential function is modifying the equilibrium between cell proliferation, differentiation, and apoptosis. It therefore plays a crucial role in promoting cancer growth, progression, invasion, and metastasis [1, 11-15]. Several studies showed that Notch1 signaling is deregulated in solid tumors such as breast, ovarian, liver, colorectal, pancreatic, and prostatic [20-24]. Nevertheless, the detailed function of Notch1 in cervical tumorigenesis is poorly characterized so far.

In the current investigation, our data unveiled the overexpression of Notch1 in cervical cancer tissues and cells as well (HeLa and SiHa). Further, we observed a strong correlation be-

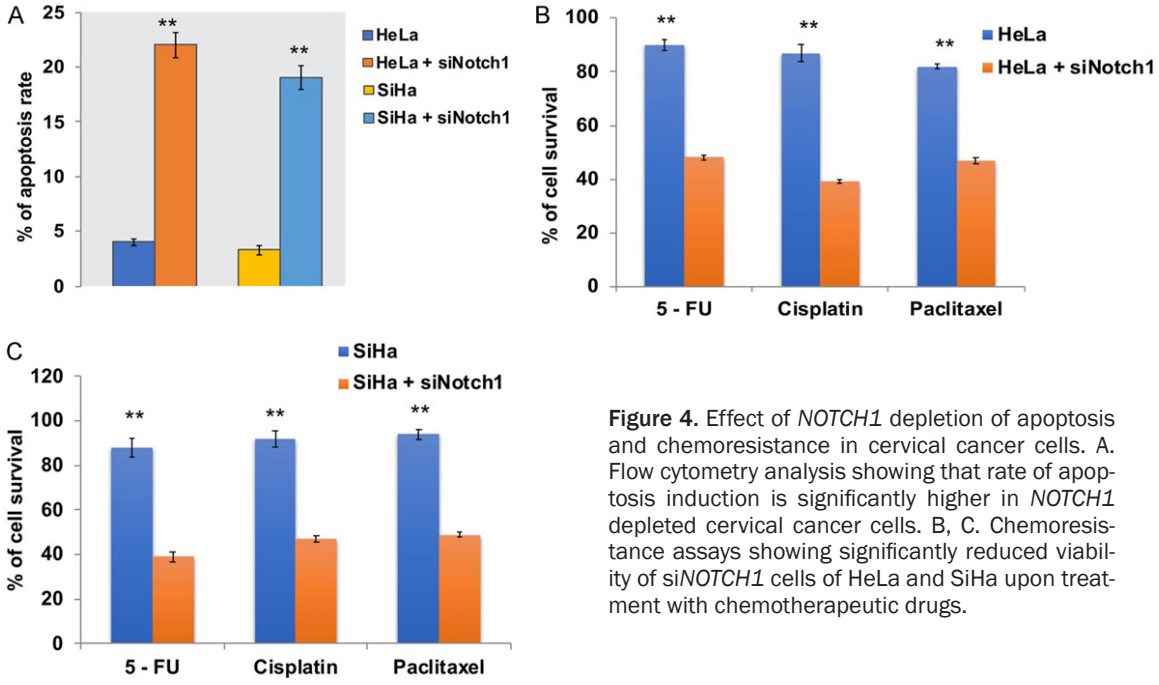

Figure 4. Effect of *NOTCH1* depletion of apoptosis and chemoresistance in cervical cancer cells. A. Flow cytometry analysis showing that rate of apoptosis induction is significantly higher in *NOTCH1* depleted cervical cancer cells. B, C. Chemoresistance assays showing significantly reduced viability of si*NOTCH1* cells of HeLa and SiHa upon treatment with chemotherapeutic drugs.

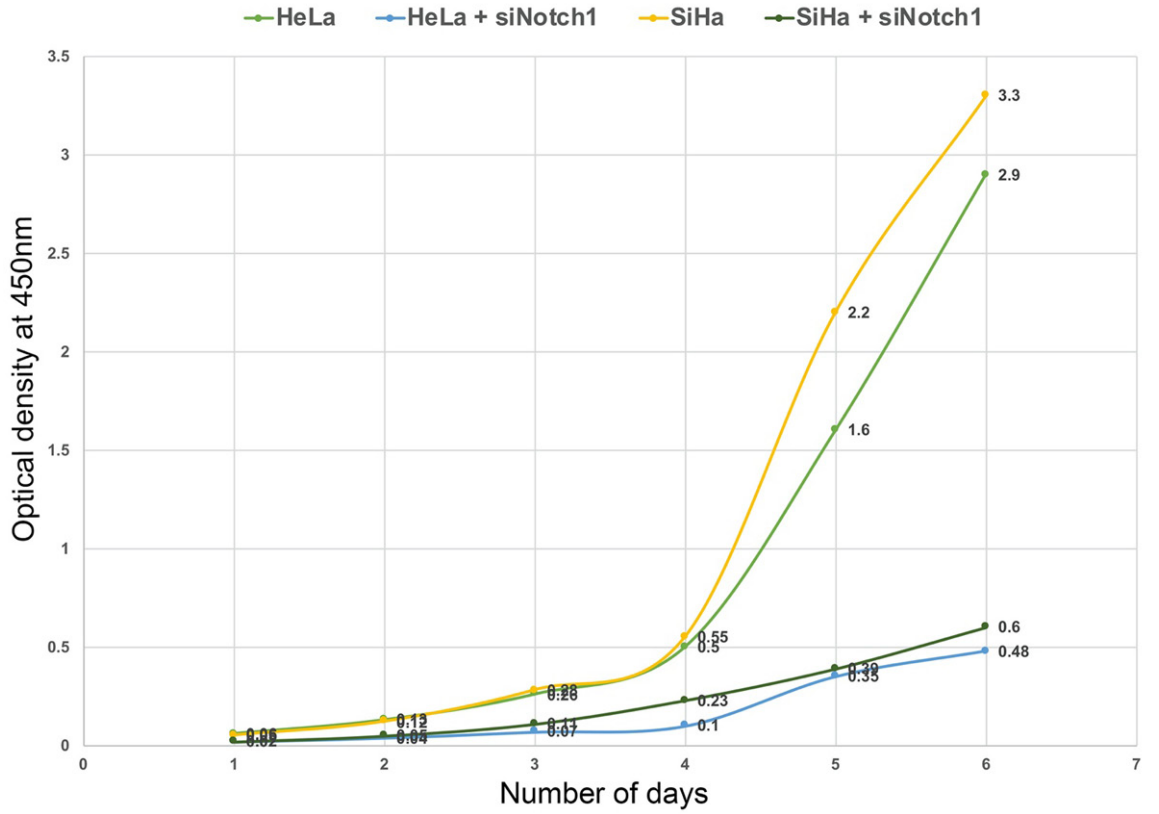

Figure 5. *In vitro* cell growth assay displaying decreased growth rate of *NOTCH1* depleted cells. Error bar: standard deviation and **; P<0.01.

tween enhanced Notch1 expression and cervical cancer tissues/cells, which suggests a prominent role of Notch1 in cervical tumorigenesis. In support of our findings, it has been pre-

viously reported that Notch1 is overexpressed in cervical cancer cells (SiHa and C33A cells) and there was a strong corelation between the Notch1 and Ki67 (cell proliferation marker) overexpression. Therefore, Notch1 plays an essential role in cell proliferation and has prooncogenic potential [1, 33]. Similarly, high signature of Notch1 signaling was involved in hepatic cellular carcinoma (HCC) and breast cancer progression [20, 27]. On the other hand, the precise function and molecular mechanism of Notch1 signaling-mediated carcinogenesis remains unclear and there are controversial outcomes in different studies [31, 34, 35]. In such instance, it has been reported that Notch-mediated cancer growth suppression was observed in association with the nuclear receptor NR4A2 [9].

Interestingly, Notch1 has been shown to protect different types of cells (neural, endothelial cells, cervical keratinocytes) from apoptosis induced by p53, drug-induced apoptosis, and different apoptotic stimuli [36, 37]. Therefore, we hypothesize that Notch1 might assist in apoptosis resistance of cervical cancer cells and performed RNAi approach to determine its role in apoptosis. Our flow cytometry analysis revealed that apoptosis was efficiently induced in si*NOCTH1* cells. As a consequence, *NOTCH1* depleted cells are highly sensitive to chemotherapeutic drugs because of apoptosis induction and cell death. Upon treatment with the drugs, the viability of the *NOTCH1* depleted cells was significantly reduced compared to the corresponding controls. These data suggest that Notch1 might be involved in apoptosis inhibition of cancer cells and thus may be one of the reasons for enhanced cancer cell proliferation. One possible explanation could be that aberrantly activated Notch might stimulate the PI3/Akt signaling through increase the level of Akt phosphorylation which leads to suppression of PTEN and tumor suppressor genes, involved in regulation of cell proliferation and apoptosis. Further, the apoptotic pathway might be compromised and cancer cells became apoptotic/multi-drug resistant owing to the deregulation of tumor suppressor genes. As a result, cell cycle regulation is disrupted and the rate of apoptosis is reduced. This leads to enhanced cell proliferation and escape from apoptotic cell death. However, the molecular mechanism behind apoptosis protection remains elusive. Other possibilities might be that the over expression of Notch1 activates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway which is associated with several antiapoptotic functions by the up-regulation of the antiapoptotic proteins Bcl-2 and Bcl-xL. This prevents apoptosis [27]. The receptor and ligands for Notch1 are single transmembrane proteins, bind with the neighbour cells upon activation and therefore Notch1 is involved in cross talk with other signaling pathways such as PI3K/AKT, NF-κB, integrins, JNK, and miRNAs for the regulation of cell fate [38]. A very recent report suggested that Numb splicing was involved in the development of cervical cancer mediated by activated Notch signaling and therefore Numb splice variants could be used as a clinical marker for cervical cancer diagnosis [39, 40].

Summary findings from this study might be helpful for future studies about Notch1 downstream targets and its functional role in apoptosis attenuation. Also, more *in vitro* apoptotic assays and animal model experiments should be performed to elucidate the role of Notch1 in apoptosis inhibition. We unveiled the prominent function of Notch1 in the tumorigenesis of cervical cancer and chemo/apoptosis resistance. Nonetheless, further detailed research must elucidate the underlying molecular mechanism of Notch1-mediated apoptosis resistance of cervical cancer cells, which would provide insight for the development of anti-cancer drugs for Notch1 intervention in cancers.

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Disclosure of conflict of interest

None.

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