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# Nedd4-1 as a Proliferating Factor in Uterine Cervical Carcinogenesis

**Soohyun Oh, Jin Woo Shin***

Department of Obstetrics and Gynecology, Gil Medical Center, College of Medicine, Gachon University, Incheon, Korea
Email: *jwshin@gilhospital.com

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## Abstract

**Objective:** Nedd4-1 has been reported to be overexpressed in various human cancers. In this study, we investigated the role of Nedd4-1 in carcinogenesis and prognosis of cervical cancer. **Methods:** We reviewed formalin-fixed paraffin-embedded tissue blocks from radical hysterectomy for cervical cancer from 2007 to 2008. A total of 49 paraffin blocks were included if they had either normal epithelium or cervical intraepithelial neoplasia (CIN) III along with cancer tissue in the same blocks. Immunohistochemical staining was performed, and overexpression was defined by both intensity and proportion of positive cells. The patients were retrospectively followed up for assessing progression-free survival (PFS) and overall survival (OS) in relation to Nedd4-1 expression. Additionally, to investigate the effect of Nedd4-1 expression in cervical cells, MTT assay and flow cytometry were used for HeLa and CaSki cells treated with Nedd4-1 and control siRNA. **Results:** Among 49 blocks, overexpression of Nedd4-1 was observed in 15.6% of normal epithelium, 37.1% of CIN III, and 79.6% of cancer and the differences were statistically significant ($p < 0.001$). Silencing Nedd4-1 decreased the cancer cell viability; however, the apoptosis rates were not associated with Nedd4-1 downregulation. During median of 92.72 months of follow-up, PFS and OS were similar between patients with and without Nedd4-1 overexpression in normal epithelium and cancer tissues ($p = 0.369$ and 0.788, and $p = 0.165$ and 0.523, respectively). **Conclusions:** Overexpression of Nedd4-1 is possibly associated with carcinogenesis of cervical cancer. Nevertheless, its prognostic significance needs to be investigated further.

## Keywords

Apoptosis, Carcinogenesis, Cervical Cancer, Nedd4-1, Proliferation

## 1. Introduction

Cervical cancer is the fourth most common cancer and the fourth leading cause of death in female worldwide [1]. Although the incidence of cervical cancer has decreased from 2013 to 2016 in Korea, 3500 new cases and 845 deaths still occurred in 2018 [2] [3]. Furthermore, the recurrence rates are 10% - 18% in patients, even in early-stage disease [4] [5]. Therefore, it is necessary to investigate mechanism of carcinogenesis and potential treatment targets for improving survival in cervical cancer patients.

Neural precursor cell-expressed developmentally down-regulated (Nedd) 4-1, a member of the E3-ubiquitin ligase family, is found in various tissues and is suggested to engage in many cellular functions [6]. It contains C2 domain, 4 WW domains and homologous to the E6-associated protein (E6-AP) carboxyl terminus (HECT) domain. The C2 domain controls Nedd4-1 binding to the membrane and recognition of substrates and the WW domain mediates protein-protein interaction with the substrate protein. Finally, the HECT domain carries the enzymatic activity of Nedd4-1 [7]. Failure of Nedd4-1 to down-regulate sodium channel activity can result in Liddle's syndrome, characterized by hypertension and hypokalemic metabolic alkalosis [8]. Nedd4-1 also participates in development of nervous system by binding and ubiquitination of fibroblast growth factor receptor 1 [9].

Researchers have investigated the function of Nedd4-1 in human carcinogenesis and progression. Overexpression of Nedd4-1 is discovered in colorectal, bladder, and gastric cancer [10]-[12]. By the way, as the name implies, the HECT domain is homologous with human papilloma virus (HPV) oncoprotein E6-AP, and E6-AP is important for inducing cervical cancer [13]. However, little is known about Nedd4-1 expression and its role in cervical cancer. We performed this study to assess the role of Nedd4-1 in carcinogenesis of cervical cancer and find its prognostic value.

## 2. Methods

### 2.1. Study Population

We collected the formalin-fixed paraffin-embedded (FFPE) tissue blocks of patients who underwent radical hysterectomy as primary treatment of cervical cancer in a tertiary referral hospital, Gil Medical Center, from January 2007 to December 2008. To reduce the errors during histologic interpretation of Nedd4 expression, we included FFPE blocks that contained normal epithelium, cervical intraepithelial neoplasia (CIN) III, and cancer in the same slide simultaneously. Patients were also eligible if the block had either normal epithelium or CIN III, along with cancer at the same time. The Gil Medical Center Institutional Review Board has approved the study (GAIRB2022-359). The informed consent was exempted due to the retrospective nature of this study.

The medical record of eligible patients was reviewed, and following items were

analyzed: age; International Federation of Gynecology and Obstetrics (FIGO) stage; type of operation; involvement of lymph node, parametrium, and resection margin; tumor size; lymphovascular space invasion; adjuvant treatment; date of operation, recurrence, death, and last visit. FIGO stage was revised in 2009 and 2018, and the stage was determined by both staging systems [14] [15]. Progression-free survival (PFS) and overall survival (OS) were defined as the interval from the date of radical hysterectomy to the date of recurrence, death, or last follow-up.

## 2.2. Immunohistochemical Staining

Immunohistochemical staining for Nedd4-1 was performed using streptavidin-peroxidase assay according to the manufacturer's instructions. The paraffin tissue sections (4-μm) were deparaffinized, and endogenous peroxidase was inactivated with 3% $H_2O_2$. For antigen retrieval, deparaffinized sections were immersed in 10 mmol/L citrate buffer and heated at 99˚C - 100˚C for 30 minutes. Then, the sections were incubated sequentially with primary anti-Nedd4-1 antibody (Millipore, Billerica, MA) at 1:100 dilution for 60 minutes, second antibody for 10 minutes, and horseradish peroxidase-streptavidin conjugate for 30 minutes. The slides were stained with 3,3'-diaminobenzidine staining and counterstained with hematoxylin and mounted.

A specialized gynecologic pathologist evaluated Nedd4-1 expression by grading the intensity of cytoplasmic staining. Immunostaining intensity was scored as 0 (negative), 1 (weak), or 2 (moderate/strong), and overexpression was defined according to both intensity and proportion of stained cells, as summarized in **Table 1**. All immunostaining scores were evaluated by this single experienced gynecologic pathologist, thereby minimizing inter-observer variability.

**Table 1.** Criteria for Nedd4-1 overexpression.

| Intensity score | Proportion of positive cells | Classification of expression |
|---|---|---|
| 0 (negative) | Any | No overexpression |
| 1 (weak) | <30% | No overexpression |
| 1 (weak) | ≥30% | Overexpression |
| 2 (moderate/strong) | Any | Overexpression |

## 2.3. Cell Cultures and Knockdown of Nedd4-1 by siRNA

The human cervical cancer cell lines, HeLa and CaSki were purchased from Korean Cell Line Bank and maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS, Welgene, Inc.), 1% (v/v) penicillin and streptomycin at 37˚C in a humidified incubator with 5% $CO_2$.

HeLa and CaSki cells were transfected using the Lipofectamine RNAiMAX (Invitrogen) transfection reagent as indicated in the manufacturer's protocol. Two different siRNAs for Nedd4-1 (Bioneer Corporation) were used, and the sequences were as follows: siRNA#1, 5'-UAGAGCCUGGCUGGGUUGUUU-3' and

siRNA#2, 5'-UUCAAUUGCCAUCUGAAGUUUAUCC-3'. A commercial scrambled siRNA was used as a negative control (Bioneer Corporation).

### 2.4. Western Blot Analysis

Cells were harvested and lysed with RIPA lysis buffer containing protease inhibitors. Protein concentration was measured using the BCA-kit. The samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were incubated in the primary antibody solution overnight at 4˚C and horseradish peroxidase-conjugated secondary antibody solution for 1 hour at room temperature. Primary antibodies include PTEN (no. SC-6817-R), actin (no. SC-1615), and Nedd4-1 (no. SC-25508). Goat anti-mouse IgG-HRP (no. SC-2005) and mouse anti-rabbit IgG-HRP (no. SC-2357) were used as secondary antibodies. All primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. A chemiluminescence reagent (Takara Bio Inc.) was applied and the chemiluminescent signals were captured by Amersham Imager 600 (GE Healthcare Biosciences AB).

### 2.5. MTT Assay

MTT assay was used to estimate the number of viable cells following transfection with Nedd4-1 and negative control siRNAs. The cells were plated at a density of 2000 or 7000 cells per well in 96-well plates and incubated for 4 hours in media with 0.5 mg/ml MTT (Sigma-Aldrich) after transfection with siRNA. Optical absorbance at 570 nm was recorded by a microplate reader.

### 2.6. Flow Cytometry

Annexin V-FITC/PI staining method was used to quantify apoptotic cell according to the manufacturer's instructions (FITC annexin V apoptosis detection kit I, BD Pharming). Briefly, siRNA-transfected cells ($5 \times 10^5$ cells in 100 mm culture dish) were washed in cold phosphate-buffered saline, suspended in binding buffer, and stained with Annexin V-FITC. The stained cells were analyzed by FACSCalibur (BD Bioscience).

### 2.7. Statistical Analysis

Results were shown as mean ± standard deviations or frequencies (%). Proportions of Nedd4-1 overexpression were compared between normal, CIN III, and cancer, and between histologic subtypes of cervical cancer using chi-square test or Fisher's exact test. Kaplan-Meier survival curve was performed for analyzing PFS and OS according to Nedd4-1 overexpression in normal epithelium and cancer.

All experiments using cervical cancer cell lines were repeated more than three times. Independent t-tests and Mann-Whitney U tests were used to compare results between cells transfected with Nedd4-1 siRNA and negative control. $p < 0.05$ was considered to be statistically significant. The statistical analysis was performed with SPSS version 21 (IBM Corp., Armonk, NY, USA).

## 3. Results

### 3.1. Patients

A total of 57 tissue blocks were identified from patients who received radical hysterectomy for cervical cancer from 2007 to 2008. Of them, 49 blocks met the inclusion criteria: 49 slides had cancer tissue, 35 slides had CIN III, 45 slides had normal epithelium, and 31 blocks had all three of them. The baseline characteristics are shown in Table 2. The histologic subtypes included squamous cell carcinoma (SCC, n = 40, 81.6%), adenocarcinoma (AC, n = 7, 14.3%), and adenosquamous carcinoma (ASC, n = 2, 4.1%). According to 2009 FIGO staging system, most of patients were stage IB (n = 43, 87.8%), and by 2018 FIGO staging, 26 patients (59.2%) were diagnosed as IB. Lymph node metastasis was found in 10 (20.4%) patients. Involvement of parametrium and resection margin were diagnosed in 8 (16.3%) and 4 (8.2%) patients, respectively. Seventeen patients did not receive any adjuvant therapy, while others received chemoradiation, radiation, or chemotherapy after operation.

Table 2. Baseline characteristics (n = 49).

| Characteristics | Total (n = 49)[a] |
|---|---|
| **Age** | 50.5 ± 11.4 |
| **Histology** | |
| Squamous cell carcinoma | 40 (81.6) |
| Adenocarcinoma | 7 (14.3) |
| Adenosquamous carcinoma | 2 (4.1) |
| **2009 FIGO stage** | |
| IA | 3 (6.1) |
| IB | 43 (87.8) |
| IIA | 3 (6.1) |
| **2018 FIGO stage** | |
| IA | 5 (10.2) |
| IB | 26 (59.2) |
| II | 5 (10.2) |
| III | 10 (20.4) |
| **Tumor size** | |
| <4 cm | 25 (51.0) |
| ≥4 cm | 24 (49.0) |
| **Lymphovascular space invasion** | |
| Negative | 20 (40.8) |
| Positive | 29 (59.2) |

Continued

| | |
|---|---|
| **Lymph node metastasis** | |
| Negative | 39 (79.6) |
| Positive | 10 (20.4) |
| **Parametrial invasion** | |
| Negative | 41 (83.7) |
| Positive | 8 (16.3) |
| **Resection margin** | |
| Negative | 45 (91.8) |
| Positive | 4 (8.2) |
| **Adjuvant treatment** | |
| No | 17 (34.7) |
| CCRT | 13 (26.5) |
| RT | 8 (16.3) |
| CT | 11 (22.4) |

[a]Values are presented as mean ± standard deviation or number (%). Abbreviations: FIGO: International Federation of Gynecology and Obstetrics; CCRT: Chemoradiation; RT: Radiation; CT: Chemotherapy.

### 3.2. Expression of Nedd4-1 by Immunohistochemical Staining

Nedd4-1 overexpression was observed in 15.6% of normal epithelium, 37.1% of CIN III, and 79.6% of cancer. The differences in Nedd4-1 overexpression were statistically significant between normal epithelium and CIN III ($p = 0.027$), between normal epithelium and cancer ($p < 0.001$), and between CIN III and cancer ($p < 0.001$). Additionally, the difference was significant between all three types of tissue ($p < 0.001$) (Table 3, Figure 1).

Table 3. Nedd4-1 overexpression in different types of tissue.

| Tissue type | Nedd4-1 overexpression[a] | | Total | p-value[b] |
|---|---|---|---|---|
| | Negative | Positive | | |
| Normal | 38 (84.4) | 7 (15.6) | 45 | |
| CIN III | 22 (62.9) | 13 (37.1) | 35 | <0.001 |
| Cancer | 10 (20.4) | 39 (79.6) | 49 | |

[a]Values are presented as number (%). [b]p-value between normal and CIN III = 0.027; CIN III and cancer < 0.001; normal and cancer < 0.001; Overall < 0.001. p-values were calculated using chi-square test. Abbreviations: CIN: Cervical intraepithelial neoplasia.

Nedd4-1 overexpression was similar between AC and ASC, between SCC and ASC ($p = 0.444$ and 1.000, respectively). However, significant differences were observed between SCC and AC ($p = 0.018$) and between all three histologic types ($p = 0.033$) (Table 4).

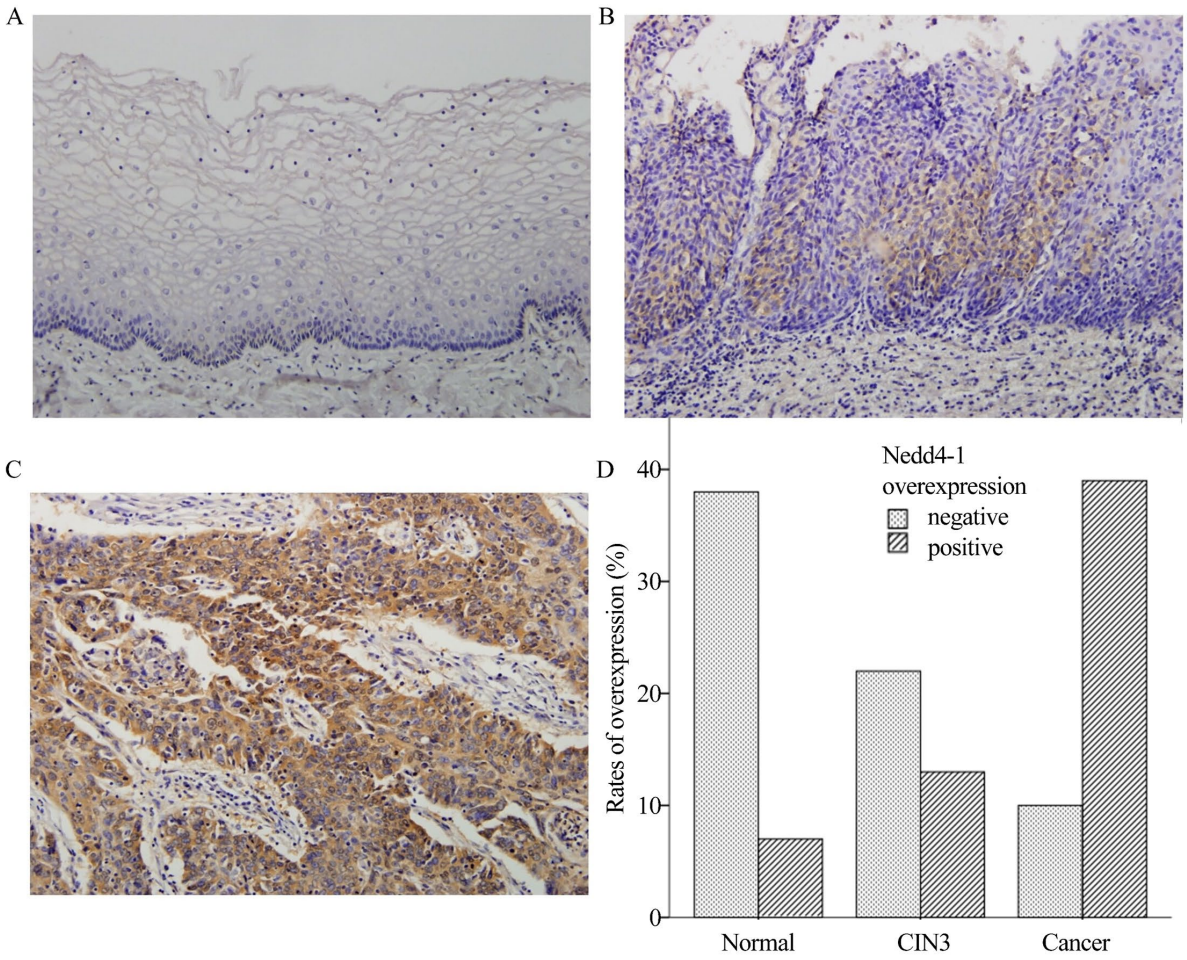


**Figure 1.** Visualization of Nedd4-1 expression in (A) normal epithelium, (B) cervical intraepithelial neoplasia (CIN) III, and (C) invasive cancer by immunohistochemistry. (D) The rates (%) of Nedd4-1 overexpression in normal epithelium, CIN III, and cancer.

**Table 4.** Nedd4-1 overexpression in histologic subtypes of cervical cancer.

| Histology | Nedd4-1 overexpression[a] | | Total | p-value[b] |
|---|---|---|---|---|
| | Negative | Positive | | |
| SCC | 5 (12.5) | 35 (87.5) | 40 | |
| AC | 4 (57.1) | 3 (42.9) | 7 | 0.033 |
| ASC | 0 (0) | 2 (100) | 2 | |

[a]Values are presented as number (%). [b]p-value between SCC and AC = 0.018; AC and ASC = 0.444; SCC and ASC = 1.000; Overall = 0.033. p-values were calculated using Fisher's exact test. Abbreviations: SCC: Squamous cell carcinoma; AC: Adenocarcinoma; ASC: Adenosquamous carcinoma.

### 3.3. Survival

Patients were followed up for median of 92.72 months (range, 0.80 - 183.80 months), and 7 patients were diagnosed with recurrence. No statistically significant differ-

ences in PFS were observed between patients with and without Nedd4-1 overexpression in both normal epithelium and cancer tissue (p = 0.369 and 0.788, respectively). Four patients died during follow-up, and OS was also similar between patients according to Nedd4-1 overexpression in both normal epithelium and cancer tissue (p = 0.165 and 0.523, respectively) (Figure 2).

### 3.4. Cell Viability by Nedd4-1 Downregulation

Western blot assay was used to evaluate Nedd4-1 and PTEN expression in control and Nedd4-1 siRNA-transfected cells (Figure 3(A)). Silencing Nedd4-1 was not related to PTEN expression. As shown in Figure 3(B), MTT assay was performed to examine the viability of cervical cancer cell upon silencing Nedd4-1. Downregulation of Nedd4-1 significantly decreased cell viability in both HeLa and CaSki cells, compared to negative control (p < 0.05).

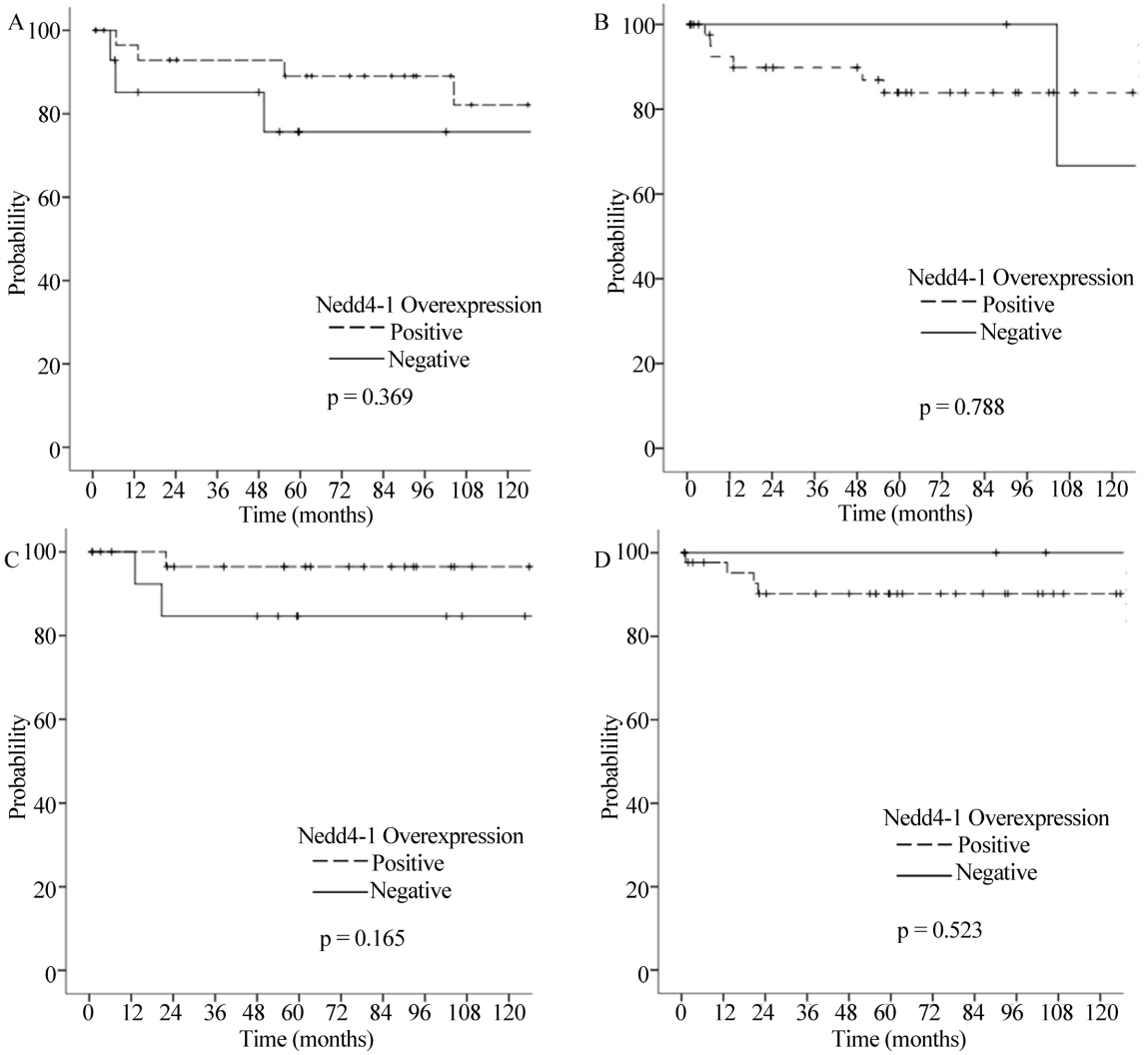


Figure 2. Kaplan-Meier survival curve of patients with or without Nedd4-1 overexpression. Progression-free survival in (A) normal cervical epithelium and (B) invasive cancer; Overall survival in (C) normal cervical epithelium and (D) invasive cancer.

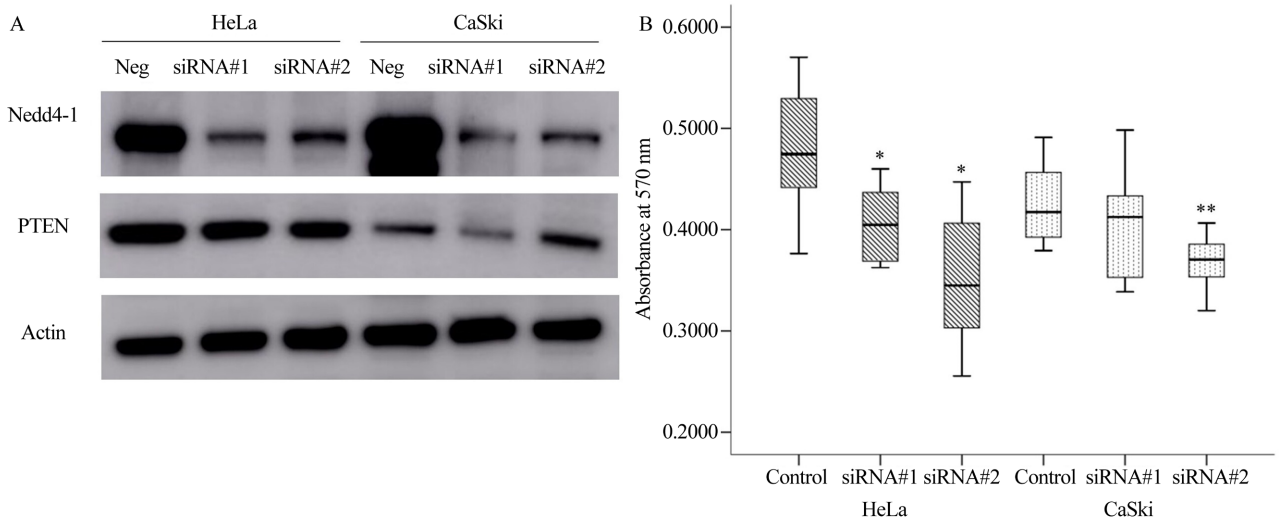


**Figure 3.** Diminished viability of cervical cancer cells, Hela and CaSki, by Nedd4-1 silencing. (A) Western blots showing Nedd4-1 downregulation by siRNA transfection. (B) The viability of cells transfected with control and Nedd4-1 siRNA. The error bars represent 95% confidence intervals. *p < 0.05, **p < 0.01 vs. negative control.

## 3.5. Apoptosis Assay Using Flow Cytometry

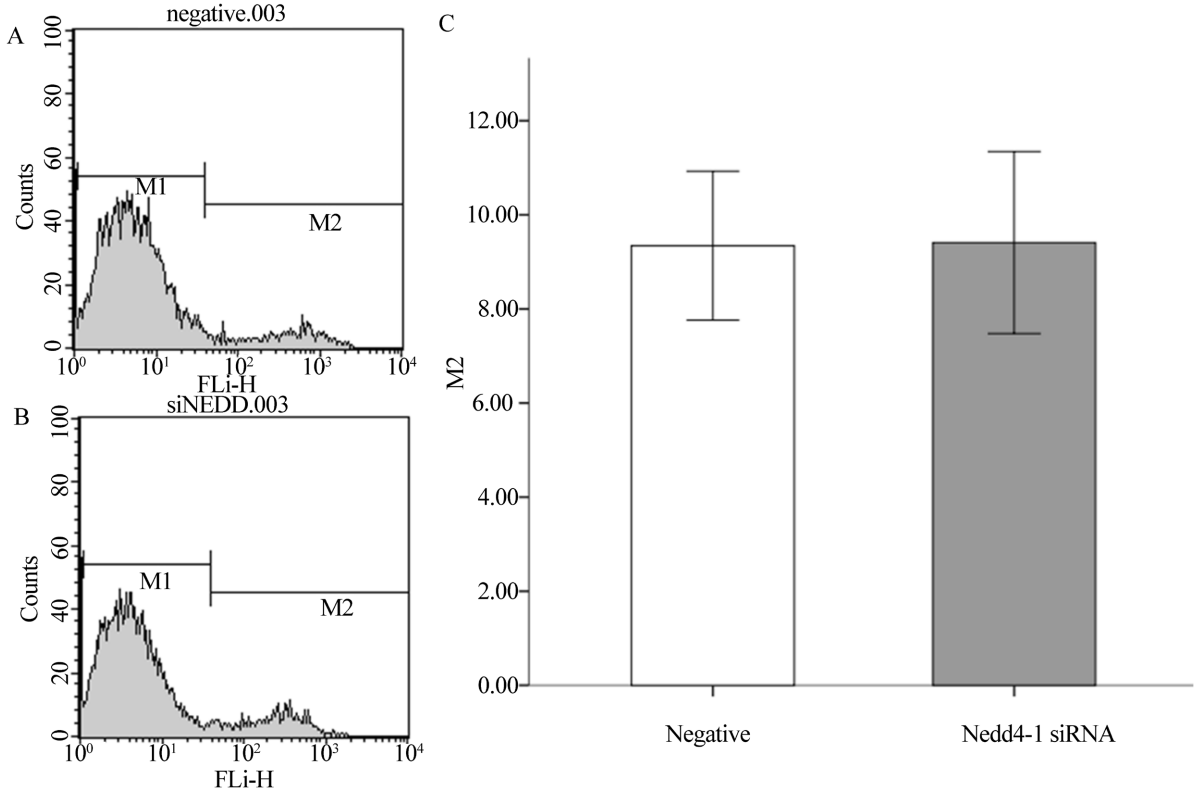


**Figure 4.** Effect of Nedd4-1 downregulation on apoptosis as observed by flow cytometry. Representative images of flow cytometry analysis of (A) negative control and (B) Nedd4-1 siRNA-transfected cells. (C) The bar graph shows the percentage of cells undergoing apoptosis in control and Nedd4-1 siRNA-transfected cells.

Flow cytometry was performed to assess apoptosis following transfection. **Figure**

4(A) and Figure 4(B) describe the number of apoptotic cells in negative control and siRNA-transfected cells. The proportion of apoptotic cells was 9.3% and 9.4%, respectively, and no differences were found (Figure 4(C)).

## 4. Discussion

In this study, we discovered the progressively increasing rates of Nedd4-1 overexpression from normal cervix epithelium through CIN III to cancer tissue. Additionally, the viability of cervical cancer cells decreased significantly by downregulation of Nedd4-1. But, Nedd4-1 downregulation was not related to apoptosis, and overexpression of Nedd4-1 was not associated with survival in this study population.

We observed increasing rates of Nedd4-1 overexpression from normal epithelium, CIN III, to cancer tissue. This result suggests Nedd4-1 overexpression is probably important in cervical carcinogenesis. In this study, three pairwise tissue-type comparisons were performed. Although no formal multiple-comparison correction was applied, all observed p-values were <0.001 and thus would remain significant even under a Bonferroni-adjusted threshold ($p < 0.0167$). The HECT domain, C-terminal region of Nedd4-1, was discovered to be like HPV E6-AP. E6-AP mediates binding of HPV E6 protein and p53, which results in degradation of tumor suppressor p53. This is one of the major mechanisms of HPV-induced cervical carcinogenesis [13] [16]. However, due to the retrospective design of this study, HPV status of the tumor tissues could not be determined. This represents a limitation, as a potential association between Nedd4-1 and HPV E6-AP has been hypothesized.

While multiple recent studies have investigated other members of the NEDD4-family ligases in cervical cancer, to our knowledge, no study has specifically examined NEDD4-1 in cervical cancer tissues. For instance, HECW1 was recently shown to suppress cervical cancer cell growth by ubiquitinating dishevelled-1 (DVL1) and inhibiting Wnt/$\beta$-catenin signaling [17]. The E3 ubiquitin ligase ITCH was reported to promote connexin43 degradation, leading to loss of gap junction communication and potentially facilitating tumor progression [18]. In addition, a novel HSP90-WWP1/WWP2-NEDD4L axis was identified as a determinant of radioresistance in cervical cancer, highlighting the role of NEDD4-family ligases in therapeutic response [19]. Our study is therefore the first to specifically evaluate NEDD4-1 expression in cervical cancer tissues, adding novel evidence to this expanding field.

Previous studies highlighted that Nedd4-1 inhibits PTEN functions by ubiquitin-mediated PTEN degradation [10] [11]. PTEN downregulation in cervical cancer cells resulted in cell-cycle arrest and apoptosis [20]. On the contrary, this study showed cancer cell viability was diminished upon silencing Nedd4-1, while the level of PTEN expression and apoptosis of cervical cancer cells remained similar. Several studies suggested oncogenic activity of Nedd4-1 through PTEN-independent mechanisms. PTEN level did not change in colon cancer cells depleted of Nedd4-1 [21]. Knockdown of Nedd4-1 in gastric cardia adenocarcinoma led to impaired

proliferation, as well as reduced invasion and migration [22].

Recent studies have demonstrated that Nedd4-1 contributes to cancer cell viability through additional non-apoptotic mechanisms. For example, Nedd4-1 facilitates proliferation by destabilizing the cell-cycle regulator p21 [23]. Nedd4-1 also stabilizes Mdm2 through K63-linked ubiquitination, which decreases p53 levels and impairs p53-mediated cellular response, thereby further promoting tumor progression [24]. In bladder cancer, Nedd4-1 enhances cell growth and migration by stabilizing Kruppel-like factor 8 (KLF8), a transcription factor that suppresses miR-132 and subsequently upregulates nuclear factor E2-related factor 2 (NRF2) [25]. These results suggest that the reduced viability observed after Nedd4-1 silencing in our study may reflect disruption of proliferation and cell-cycle regulation. Further studies are warranted to clarify how Nedd4-1 acts as an oncoprotein in cervical cancer.

Overexpression of Nedd4-1 was shown more frequently in SCC than in AC in this study. On the other hand, a study of non-small cell lung cancer reported that the rates of Nedd4-1 overexpression were higher in SCC (61.0%) than that in AC (52.2%) and the difference was not significant [11]. Moreover, Nedd4-1 expression was comparable among different histologic subtypes and differentiation in colorectal and gastric cancer tissues [12]. These inconsistent results imply possibility of histology-specific behavior of Nedd4-1 in cervical cancer.

Although there was trend of worsening prognosis in patients with Nedd4-1 overexpression in cancer tissue, no significant association was noted between presence of Nedd4-1 overexpression in either normal or cancer tissue and survival. Similarly, other studies found that Nedd4-1 overexpression did not relate to any clinicopathologic characteristics, such as histologic subtypes, depth of invasion, or metastasis in colorectal, gastric, and lung cancers [11] [12]. However, a study conducted in patients with gastric cardia adenocarcinoma presented that 5-year survival rates significantly decreased in patients with Nedd4-1 overexpression [22]. In this study, the number of participants was not enough to confirm the prognostic value of Nedd4-1 overexpression in cervical cancer. The performance of Nedd4-1 as prognostic marker should be examined in a larger population.

## 5. Conclusion

In conclusion, the present study suggested for the first time that overexpression of Nedd4-1 may promote cervical cancer development. There is a need for more studies to establish the specific mechanism of oncogenic role of Nedd4-1.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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