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Clinical Significance of Ubiquitin-associated Protein 2-like in Patients With Uterine Cervical Cancer

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Abstract. *Background: Ubiquitin-associated protein 2-like (UBAP2L) has been demonstrated to be associated with the progression of multiple types of cancer. However, the function of UBAP2L in uterine cervical cancer remains unclear. Materials and Methods: Between 2005 and 2015, 84 patients who underwent surgery were included in this study. The patients were stratified into two groups on the basis of immunohistochemical staining for UBAP2L, and survival analysis was performed. Moreover, loss-of-function analysis was performed using the cervical cancer cell lines CaSki and SiHa. Results: Based on immunohistochemistry, the overall survival in patients with low UBAP2L expression was significantly longer than that of those with high UBAP2L expression (p=0.045). The in vitro experiment revealed that knockdown of UBAP2L remarkably inhibited cell proliferation in both live cell imaging and the MTS assay. Conclusion: Patients with high UBAP2L expression had unfavorable prognosis and UBAP2L appears to play an important role in proliferation.*

Cervical cancer is a common malignancy observed among females worldwide. The mortality rates associated with uterine cervical cancer have declined due to the widespread use of cancer screening for the prevention and early detection of cervical cancer (1). However, approximately one-third of patients experience recurrence within 5 years, and their post-recurrence oncological outcome is generally poor (2-4). Therefore, the oncological outcome for these patients is far from satisfactory.

Ubiquitin-associated protein 2-like (UBAP2L), also known as NICE-4, is a widely conserved protein with a ubiquitin-associated domain and multiple RGG/RG repeats at the *N*-terminus. UBAP2L has also been shown to aggregate after proteasome inhibition (5). The ubiquitin-proteasome pathway is commonly used for protein degradation in eukaryotes and is considered to be involved in multiple cellular processes (6-8). In addition, recent studies have confirmed that *UBAP2L* functions as an oncogene and is associated with various types of cancer, including prostate cancer (9), glioma (10), hepatocellular carcinoma (11, 12), lung adenocarcinoma (13), and breast carcinoma (14). Therefore, inhibition of the ubiquitin-proteasome system is a promising anticancer strategy, and several proteasome inhibitors have been approved for clinicaI use (15-17). In uterine cervical cancer, oncogenesis by human papillomavirus (HPV) is mediated by ubiquitin ligase pathways alteration (6). However, the function and clinical impact of UBAP2L in uterine cervical cancer remains unknown.

In this study, we investigated UBAP2L expression in surgical specimens of cervical cancer and its effect on survival. Subsequently, we performed loss-of-function analysis and investigated the effect of *UBAP2L* knockdown on cell proliferation and cell-cycle arrest.

Materials and Methods

Patient selection and immunohistochemistry (IHC). We retrospectively reviewed all the records of patients with cervical cancer who were initially treated in our hospital from 2005 to 2015. This study was approved by the Ethics Committee of our Institute (approval no.: 2017-0053), and approval for an opt-out consent method was provided. Informed consent was obtained by the opt-out consent method on Nagoya University website. We included 84 patients who

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underwent radical hysterectomy (RH), and for whom histological examination revealed pT1b1 to pT2b squamous cell carcinoma (SCC). Patients who had received neoadjuvant therapy and had no residual tumor in the RH specimen after conization were excluded.

Archival formalin-fixed, paraffin-embedded tumor tissue obtained at RH was used in this study, and sections of 4-μm thickness were prepared using a microtome. The sections were deparaffinized, rehydrated, subjected to antigen retrieval in 10 mM sodium citrate (pH 6.0) for 20 min at 95˚C in a microwave, and treated with 0.3% hydrogen peroxide in methanol for 20 min after being washed with phosphate-buffered saline (PBS). The sections were then blocked with goat serum using Histofine SAB-PO(R) kit according to the manufacturer protocol (Nichirei, Tokyo, Japan) and incubated with a polyclonal rabbit antibody to UBAP2L (HPA035068; Sigma-Aldrich, St. Louis, MO, USA) diluted by PBS at 4˚C overnight. After rinsing with PBS, the sections were incubated with a secondary antibody, and then peroxidase-labeled streptavidin. The sections were then rinsed with PBS and developed by the 3,3'-diaminobenzidine (DAB) substrate-chromogen (Nichirei, Tokyo, Japan). After rinsing in water, the sections were incubated with hematoxylin, dehydrated, and mounted. Based on the IHC activity, a four-tiered semi-quantitative score was assigned according to the intensity and area of stained cells, as follows: The staining intensity was scored as: 0, negative; 1, weak; 2, medium; or 3, strong. The percentage of the staining area was scored as 'focal' (1-10%), 'sporadic' (11-50%), and diffuse (>51%) relative to the total tumor area (Figure 1A). All photographs were taken using Zeiss Axio Imager.A1 (Carl Zeiss, Tokyo, Japan).

The Cancer Genome Atlas (TCGA) database. Normalized RNA-Seq expression data and clinical survival information of tumor samples were downloaded from cBioPortal (http://www.cbioportal.org). Using data of 187 patients with stage I or II SCC, we performed survival analysis stratified by *UBAP2L* mRNA expression (low: z-score <0 or high: z-score ≥0).

Cell culture and small interfering RNA (siRNA) transfection. Cervical cancer cell lines, CaSki and SiHa were purchased from the American Type Culture Collection (Manassas, VA, USA). These cell lines were maintained in RPMI (Sigma-Aldrich) containing 10% fetal bovine serum (Sigma-Aldrich) and penicillin-streptomycin solution (Meiji Seika Pharma, Tokyo, Japan). These cell lines were confirmed to be negative for mycoplasma contamination.

To suppress *UBAP2L*, two different Silencer Select Pre-designed siRNAs for *UBAP2L* were used (siRNA IDs were s19176 and s230223; Thermo Fisher Scientific, Waltham, MA, USA). The sequences of s19176 and s230223 were 5''-GCAGCUUCCUUACUGACGA-3' (siUBAP2L-1) and 5'-GCAGAAUACCCUUUCAUCA-3' (siUBAP2L-2), respectively. Silencer Negative Control No. 1 siRNA (Thermo Fisher Scientific) was used as a control (siCtrl). Cells were transfected with 3 nM siRNA using Lipofectamin RNAi Max (Thermo Fisher Scientific, Waltham, MA). Cells were used for further analysis 72 h after transfection.

Western blotting. Total protein was extracted from cell lines using RIPA Protein Lysis solution (Thermo Fisher Scientific, Waltham, MA). Protein quantification was carried out using BCA protein assay kit (Wako, Osaka, Japan). After quantification, 30 μg of total proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel electrophoresis and transferred onto PVDF membranes. The transferred polyvinylidene fluoride membranes were treated with 5% skim milk for 30 min at room temperature before being incubated overnight at 4˚C with a rabbit anti-UBAP2L, purified in a previous study (18).

The membranes were washed thrice with phosphate-buffered saline with Tween 20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Finally, the immunoreactive bands were detected using enhanced chemiluminescence.

Proliferation assay. Transfected cells were reseeded in a 96-well plate at a density of 1,000 cells/well and incubated overnight. The plate was moved into an IncuCyte ZOOM (Essen BioScience, Ann Arbor, MI, USA) for live cell imaging, and images of each well were recorded every hour for a total of 72 h. Finally, the confluency of each well was determined using IncuCyte ZOOM 2018 software.

For the MTS assay, 10 μl of 5 mg/ml CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA) was added to each well and incubated with cells for 2 h. The optical density (OD) of each well was determined using a spectrophotometer at a wavelength of 490 nm.

Cell-cycle analysis. Transfected cells were reseeded in 6-cm dish and grown for 24 consecutive h. The cells were harvested following trypsinization, washed with 1% bovine serum albumin (BSA; Wako, Osaka, Japan)/PBS, and fixed in cold 70% ethanol at 4˚C overnight. The fixed cells were washed with 1% BSA/PBS and collected by centrifugation (300×*g* for 30 min). Cells were resuspended in propidium iodide and RNase solution (BD Biosciences, San Jose, CA, USA) and used for FACS Canto2 flow cytometer analysis (BD Biosciences). The obtained data were analyzed with the ModFit LT DNA analysis program (Verity Software House, Topsham, ME, USA).

Statistical analysis. Statistical analysis was performed with SPSS version 25 (IBM, Armonk, NY, USA). The baseline differences between patients stratified by UBAP2L expression were assessed by Student's *t*-tests and Mann-Whitney U-tests. Kaplan-Meier curves and log-rank test were used for the survival analysis. Three parameters of survival were studied: overall survival (OS), progression-free survival (PFS), and disease-free survival (DFS). OS was defined as the time from initial surgery to death from any cause and PFS was the time from initial surgery to tumor progression or death from any cause. In the analysis of the TCGA database, OS and DFS data were obtained from the database. For the *in vitro* assay, differences were assessed by Student's *t*-tests. A value of $p<0.05$ was taken as statistically significant.

Results

A total of 82 patients with SCC of the uterine cervix were enrolled. All the patients underwent RH without neoadjuvant therapy, and their surgical specimens were used for IHC. UBAP2L expression was strong in 21 (25.0%), moderate in 24 (28.6%), weak in 29 (34.5%), and negative in 10 (11.9%) patients. Representative IHC images are shown in Figure 1A. The association between UBAP2L expression and survival was also evaluated by stratifying patients into groups with low UBAP2L (negative and weak) and those with high UBAP2L (moderate and strong).

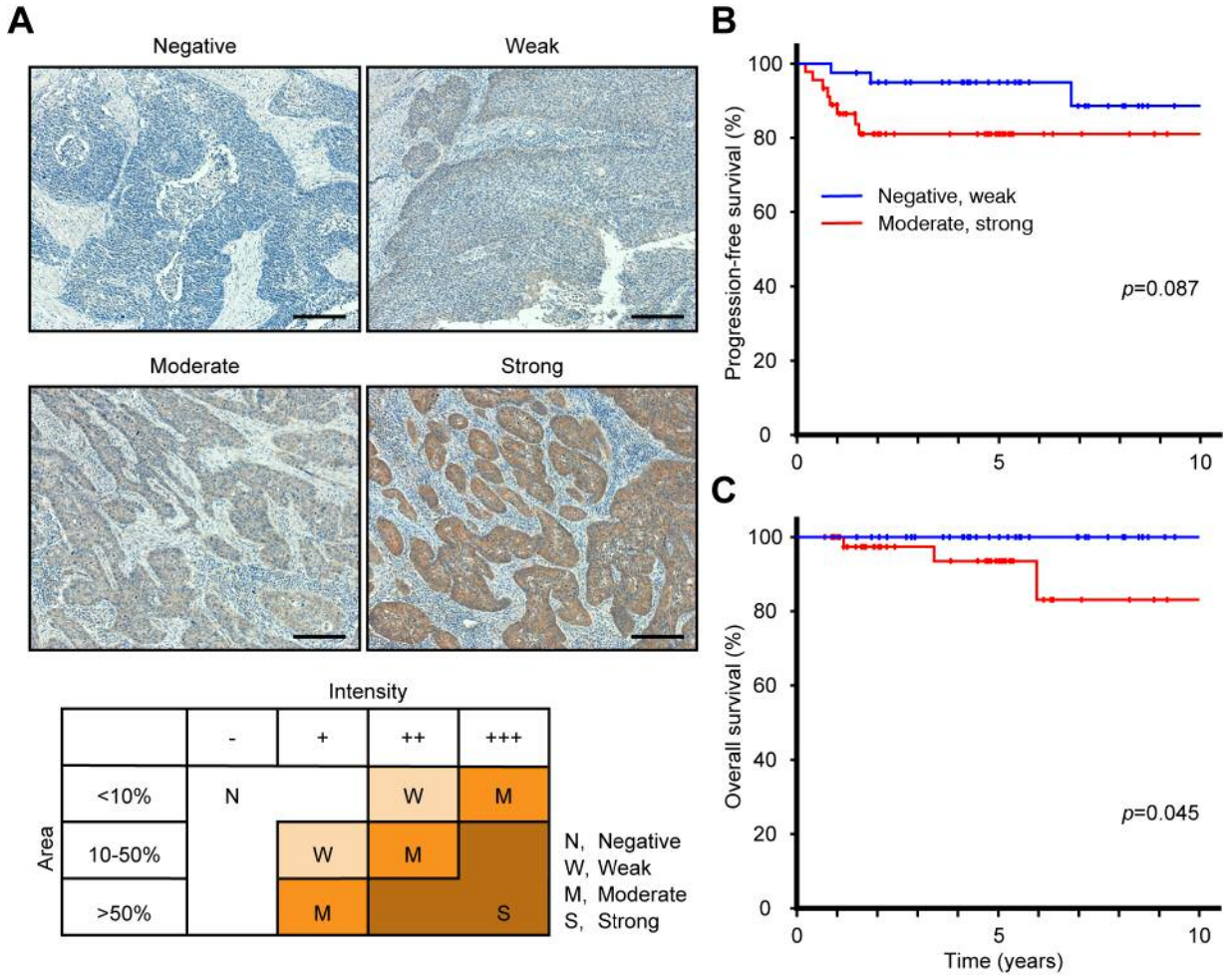

Figure 1. *Ubiquitin-associated protein 2-like (UBAP2L) expression in patients with cervical cancer and survival impact. A: Representative images of immunohistochemistry and evaluation of immunohistochemistry (Scale bars=200 μm). Kaplan-Meier curves showing progression-free (B) and overall (C) survival stratified by UBAP2L expression.*

In the 59.1-month median follow-up period (range=8.2-153.3 months), 11 patients experienced recurrence and three patients died. Patient characteristics, including age, pT, tumor size, pN, lymphovascular space invasion, and treatment, were not significantly different between the low and high UBAP2L groups (Table I). Kaplan-Meier curves indicated that PFS in the low UBAP2L group tended to be longer than that of the high UBAP2L group (p=0.087) (Figure 1B). All three patients who died were in the high UBAP2L group, and Kaplan-Meier curves revealed that the difference in OS between the two groups was significant (p=0.045) (Figure 1C).

To validate our result from IHC, we performed survival analysis using the TCGA database. According to the database, 53 patients had low *UBAP2L* mRNA expression (z-score <0) and 134 patient high expression (z-score ≥0). Kaplan-Meier curves demonstrated significantly longer DFS and OS in the low expression than the high expression group (p=0.014 and p=0.031, respectively) (Figure 2).

We next evaluated the role of UBAP2L expression *in vitro*. The efficacy of *UBAP2L* down-regulation was confirmed by western blot (Figure 3A). Representative images of live cell imaging are shown in Figure 3B, and the confluency for each image was calculated. At 72 h, the siCtrl CaSki cells had proliferated 5.69 times, while the siUBAP2L-1 and -2 CaSki cells had proliferated 2.76 and 3.60 times, respectively. Therefore, knockdown of *UBAP2L* significantly inhibited the proliferation of CaSki cells (p<0.001 for both groups, Figure 3C). Similar growth inhibition was observed in the SiHa cells. siCtrl, siUBAP2L-1, and -2 SiHa cells had proliferated 6.03, 4.92, and 3.58 times, respectively (siUBAP2L-1 and -2: p=0.002 and

Table I. *Baseline characteristics of patients with cervical cancer.*

		UBAP2L		
		Negative, weak (n=39)	Moderate, strong (n=45)	*p*-Value
Age, years	Median (range)	50 (21-68)	45 (20-73)	0.631
pT, n (%)	1b	21 (53.8%)	24 (53.3%)	0.963
	2a, 2b	18 (46.2%)	21 (46.7%)	0.844
Tumor size	<4 cm	36 (92.3%)	41 (91.1%)	0.356
	≥4 cm	3 (7.7%)	4 (8.9%)	0.161
pN, n (%)	0	28 (71.8%)	28 (62.2%)	0.512
	1	11 (28.2%)	17 (37.8%)	0.631
LVSI, n (%)	–	11 (28.2%)	7 (15.6%)	0.963
	+	28 (71.8%)	38 (84.4%)	0.844
Treatment, n (%)	RH	8 (20.5%)	12 (26.7%)	0.356
	RH + CCRT	31 (79.5%)	33 (73.3%)	0.161

LVSI: Lymphovascular space invasion; RH: radical hysterectomy; CCRT: concurrent chemoradiotherapy.

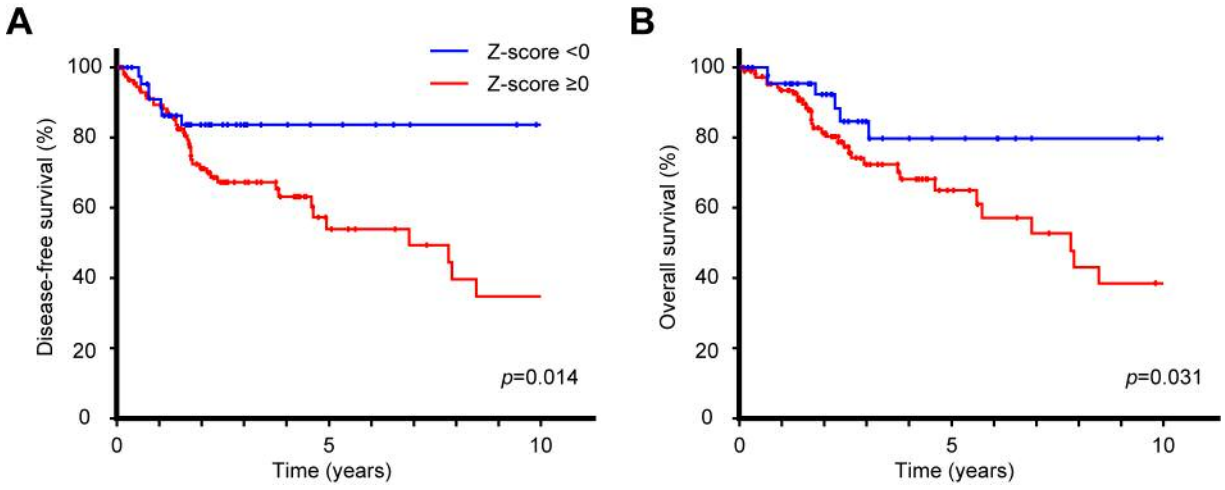

Figure 2. *Survival impact of ubiquitin-associated protein 2-like (UBAP2L) mRNA expression from the The Cancer Genome Atlas (TCGA) database. Kaplan-Meier curves showing disease-free (A) and overall (B) survival stratified by UBAP2L mRNA expression Z-score.*

p<0.001, respectively, Figure 3C). The results of the MTS assay also supported this growth-inhibitory effect. At 72 h, compared with siCtrl CaSki, the OD490 values of siUBAP2L-1 and -2 cells decreased to 65.2% and 66.8%, respectively (*p*<0.001 for both groups, Figure 3D). Moreover, compared with siCtrl SiHa cells, the OD490 values of siUBAP2L-1 and -2 cells decreased to 78.7% and 54.5%, respectively (*p*<0.001 in both groups, Figure 3D). Therefore, knockdown of *UBAP2L* significantly inhibited cell proliferation in both cell lines.

Finally, in order to elucidate the potential mechanism underlying growth inhibition, the effects of *UBAP2L* knockdown on the cell-cycle distribution were evaluated. In both cell lines, the cell population in the G_0/G_1 phase was significantly reduced (CaSki siUBAP2L-1 and -2: *p*=0.006 and *p*=0.026, respectively, and SiHa siUBAP2L-1 and -2: *p*=0.004 and *p*=0.007, respectively, Figure 4). Moreover, the cell population in the S phase was significantly increased in siUBAP2L CaSki cells (*p*=0.002), and those in G_2/M phase tended to increase in both cell lines (Figure 4).

Discussion

Previous studies demonstrated that UBAP2L is involved in the ubiquitin-proteasome system that degrades most intracellular proteins (5, 19). However, the function of UBAP2L in cervical

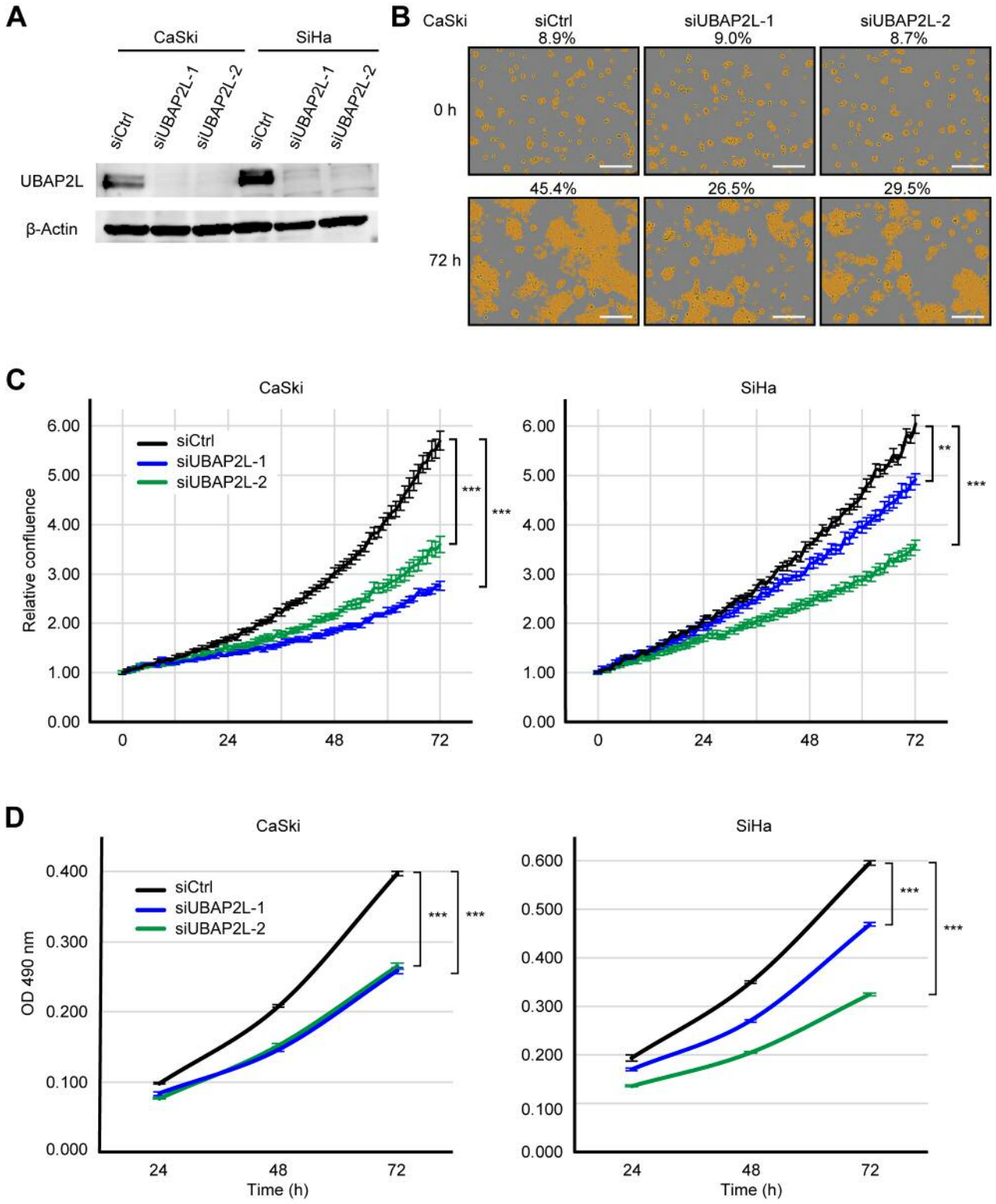

Figure 3. *Down-regulation of ubiquitin-associated protein 2-like (UBAP2L) using small interfering RNA (siRNA) inhibited the proliferation of cervical cancer cells. A: Western blot analysis of the UBAP2L protein level in the cervical cancer cell lines CaSki and SiHa. Beta actin was used as a loading control. B: Representative images of CaSki cells analyzed by IncuCyte ZOOM. The confluence values are shown (scale bars, 300 μm). C: Growth curves based on the relative confluence generated by the IncuCyte ZOOM. D: Growth curves based on MTS assay. Data represent the mean±standard error. Significantly different at **p<0.01, ***p<0.001 vs. siCtrl.*

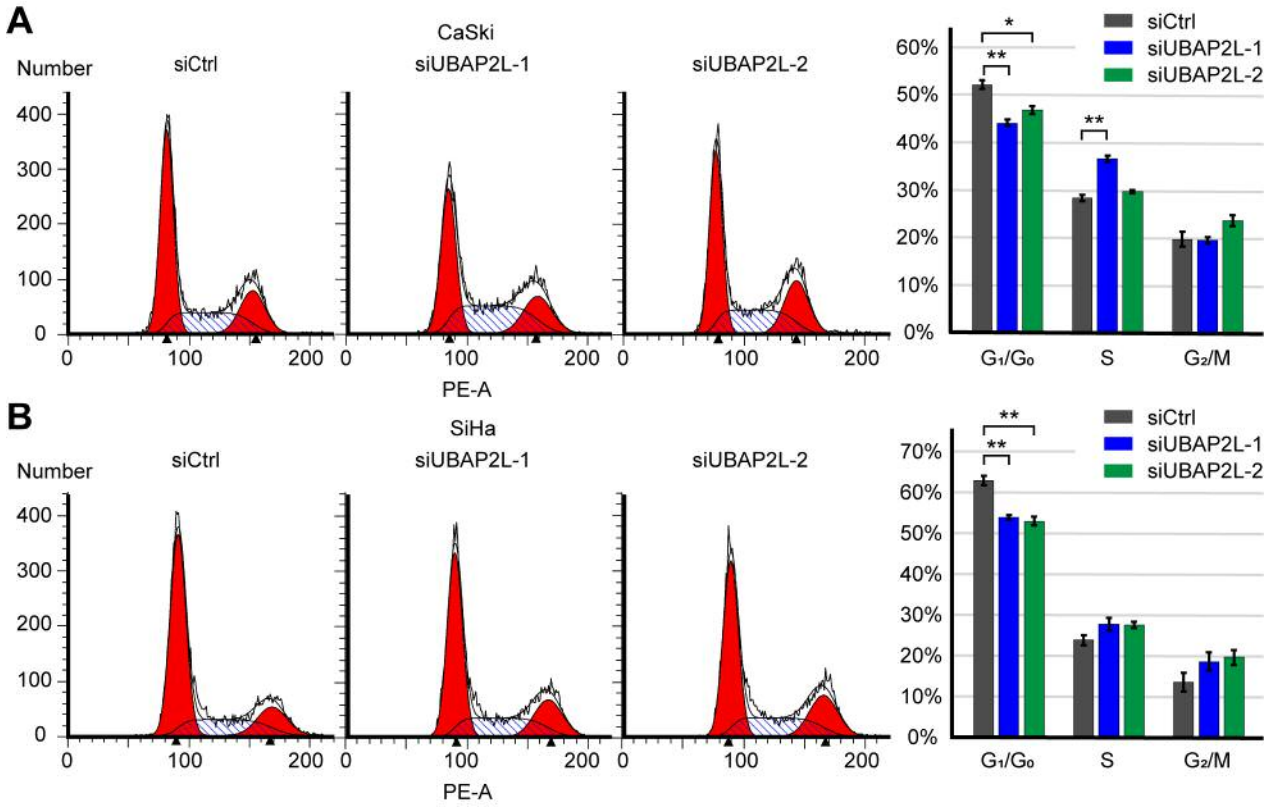

Figure 4. *Down-regulation of ubiquitin-associated protein 2-like (UBAP2L) induced cell-cycle arrest. The cell-cycle distribution of CaSki (A) and SiHa (B) cells was analyzed by flow cytometry. Data represent the mean±standard error. Significantly different at *p<0.05, **p<0.01 vs. siCtr.*

cancer has not yet been elucidated. To the best of our knowledge, the present study is the first to evaluate the impact of UBAP2L expression in cervical cancer.

Initially, IHC revealed that high expression of UBAP2L was associated with significantly poor prognosis in patients with cervical cancer. However, there were only a few patients who experienced recurrence or death in our cohort. Thus, we used data from the TCGA database to validate the poor prognosis associated with high UBAP2L expression. According to previous reports, *UBAP2L* is up-regulated in hepatocellular carcinoma compared with normal tissues, and the prognosis of patients with high UBAP2L expression is significantly poorer than those with low expression (11, 12). Moreover, a similar result was also reported in patients with lung adenocarcinoma (13). Therefore, overexpression of UBAP2L is considered to be associated with poor prognosis and to contribute to cancer progression.

In order to elucidate the function of UBAP2L in cervical cancer, loss-of-function analysis was performed *in vitro*. The results demonstrated that down-regulation of *UBAP2L* reduced proliferation and led to cell-cycle arrest in cervical cancer cell lines. These results were consistent with previous reports which used several cell lines (9, 10, 14). It has been shown that the arginine methylation of UBAP2L by protein arginine *N*-methyltransferase 1 is required for the proper distribution of chromosomes during mitosis (18). Hence, UBAP2L is considered to play an important role in mitosis; thus, the finding that down-regulation of *UBAP2L* resulted in G_2/M arrest was typical (9, 14). However, several cell lines have been shown to undergo G_0/G_1 or S phase arrest as a result of *UBAP2L* down-regulation (9, 10). Therefore, UBAP2L function might differ depending on the cell type, and UBAP2L might have additional unknown functions. In addition, differently from other malignancies in the previous reports (9-14), cervical cancer is caused by HPV, and its oncogene *E6* and *E7* target key cell-cycle control proteins (6, 20). Therefore, further evaluation of the association between UBAP2L and HPV oncogenes would be interesting.

Recently, a report indicated that UBAP2L modulates stress granule (SG) assembly (21). SGs are non-membranous cytoplasmic foci composed of non-translating messenger ribonucleoproteins (22). Various environmental stresses, such as heat shock, oxidative stress, and chemotherapeutic drugs, induce SG assembly to minimize stress-related damage and

promote cell survival, but X-ray irradiation does not (22-24). However, SG formation occurs under hypoxic conditions, and SG depolymerization, caused by radiation-induced reoxygenation, is involved in hypoxia inducible factor-1 (HIF1) activation (23-25). As a result, HIF1-regulated cytokines enhance radioresistance (25); hence UBAP2L might be associated with both chemo- and radioresistance. Therefore, not only increased proliferation but also treatment resistance due to UBAP2L might be associated with poor prognosis of patients with high UBAP2L expression, although we did not evaluate SG and UBAP2L-related treatment resistance in this study.

The present study has several limitations. Firstly, in IHC analysis, we only evaluated patients with early-stage disease because we used surgical specimens. Thus, the nature of UBAP2L expression in advanced or recurrent cervical cancer remains unknown. Secondly, as described previously, because there were only a few patients who experienced recurrence or death, the statistical power was relatively weak. Thirdly, in the TCGA database, the details of the patients are unknown, and potential bias might have existed. Therefore, further studies examining the use of UBAP2L as a prognostic factor are desired. In the *in vitro* analysis, we evaluated the impact of UBAP2L on cell proliferation and the cell cycle. Additional functions of UBAP2L, such as in SGs and epithelial-mesenchymal transition, should be evaluated in future studies.

In conclusion, we demonstrated that UBAP2L plays a critical role for proliferation in cervical cancer, and as a result, UBAP2L expression was correlated with poor prognosis. UBAP2L might be a potential therapeutic target, and further studies are desired.

Conflicts of Interest

None.

Authors' Contributions

KY contributed to acquisition of data and was a major contributor in writing the article. HK contributed to conception and design and gave final approval of the version to be published. EI contributed to acquisition of data. ST involved in revising the article critically. YI contributed analysis and interpretation of data. NY contributed to acquisition of data. KN contributed analysis and interpretation of data. FU contributed to conception and design and involved in revising the article critically. KN, SS, KS, and AN contributed analysis and interpretation of data. FK gave final approval of the version to be published. All Authors read and approved the final article.

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