Effect of PHAP1 and SUMO2 on biological characteristics of Cervical squamous cell carcinoma

Qian Liu
Xinjiang Medical University
Xiaohong Li
Xinjiang Medical University Affiliated First Hospital
Song Qin (qs551@163.com)
Xinjiang Medical University Affiliated First Hospital

Research article

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Abstract

Background This study aimed to investigate the biological characteristics of PHAP1 and SUMO2 in CSCC and the relationship between the expression of the 2 genes and HPV16 infection. Method To detect the function of PHAP1 and SUMO2 in the occurrence and development of CSCC, we first compared their expression patterns in CSCC tissue samples, CIN and matched normal tissues through IHC, and RT-PCR. In addition, we carried on WB assay to test the expression of PHAP1 and SUMO2 in the SiHa, C33A and Ect1 cell lines. We analyzed the relationship between the expression of PHAP1 and SUMO2 and HPV16 infection. Result The results demonstrated that PHAP1 and SUMO2 expression at both the protein and mRNA levels was elevated in CSCC tissues compared with CIN and normal tissues. The expression of SUMO2 was significantly associated with lymph node metastasis (P=0.02), AJCC stage(p=0.024), but not other clinicopathological factors. The expression of PHAP1 and SUMO2 protein in SiHa, C33A cells was obviously higher than that in Ect1 cells. The expression of PHAP1 and SUMO2 was associated with a susceptibility to HPV16 infections. Conclusion Our results imply that PHAP1 and SUMO2 may be potential tumor promoter genes and may provide the biological basis for diagnosis, prognosis and treatment for CSCC.

Background

Cervical carcinoma (CC) is the most common malignant tumor among gynecological cancer worldwide[1]. CC is a highly aggressive tumor and is the second most common cancer among women following breast cancer and the third highest cause of deaths in females[2]. More than 70% of cervical cancer patients were invasive once been diagnosed, among which squamous cell carcinoma (SCC) is the most frequent type[3]. Cervical squamous cell carcinoma (CSCC) affects the health of women significantly worldwide ,the high fatality of CSCC is still a public health and socioeconomic issue, especially in less-developed regions, Xinjiang, China[4]. Therefore, it is urgent to find new and effective diagnosis and treatment methods.

As we all know, most cervical cancer patients are caused by human papillomavirus (HPV) infection, which is considered to be a preventable cancer. We can find HPV DNA in almost all cervical cancer patients[5]. At present, more than 100 kinds of HPV have been identified. According to its carcinogenic potential, HPV is divided into high-risk group (hrHPV) and low-risk group (lrpv) [6]. HPV (hrHPV) types 16 and 18 strains are associated with more than 70% of CC [7]. Recently,a wide range of HPV screening programs have greatly improved the accuracy and survival rate of diagnosis in many countries [8]. The literature has reported that other factors, including the immune system and genetics of the host, as well as the viral genotype, appear to play a role in the development of cervical carcinoma[9].

Many studies have confirmed that PHAP1(HLA-DR related protein 1) is closely related to cell differentiation, gene transcription, apoptosis and cell cycle transformation. It is a nuclear phosphoprotein, also known as ANP32A (acid leucine rich nuclear phosphoprotein 32A), which plays a role in a variety of cells. It is a protein having complex function that is abnormally expressed in many human cancers [10].

However, What role does PHAP1 play in the end? Its expression pattern and clinical function in cervical cancer remain unclear.

In recent years, small ubiquitin related modifying proteins (SUMO) have been discovered which are related to various biological functions[11]. SUMO2 has the function of post-translational modification and binding to the target protein, which blongs to the SUMO family,including SUMO1, SUMO2 and SUMO3[12]. In addition, SUMO2 protein can also regulate the function of target molecules [13].

Therefore, we will used RT-PCR,IHC,WB methods to have a deep research aimed to investigate the function of PHAP1 and SUMO2 in CSCC offering a novel insight into understanding the mechanism of CSCC.

Methods

Patients and clinical samples collection

We collected fresh tissue samples by asking the results of preoperative pathological diagnosis, including 27(33.3%) CSCC cases,35(43.2%) CIN cases,19(23.5%) histologically normal cases. All fresh tissue samples were collected and stored in liquid nitrogen immediately after the operation. Meanwhile, we collected FFPE tissue samples including 40(39.2%) CSCC cases,39(38.2%) CIN cases,23(22.5%) normal cases. These tissues acquired from patients who underwent surgical resection in the First affiliated Hospital of Xinjiang Medical University between 2015 and 2018. We collected all the clinicopathological parameters of the patients in the study. The average age of the patients was 50.32 years; the youngest was 36 years old and the oldest was 76 years old at Operative time. Their mean age + standard deviation was 50.32±9.45. According to the American Joint Committee on Cancer (AJCC), tumor staging was determined, involved tumor size (T), lymph node involvement (N), and distant metastasis (M). According to AJCC, 40 patients with CSCC were divided into the following categories: pathological stage T1,33(82.5%); pathological stage T2,1(2.5%); pathological stage T3, 6(15%).Then,the following patient characteristics were collected for the 40 CSCC patients, including: age, tumor size, degree of differentiation, ethnic, lymph node status and so on. All experiments for this study were approved by the ethics committee of Xinjiang Medical University.

Cell culture

In this study,CSCC cell lines, SiHa(HPV16-positive), C33A (HPV16-negative) and Ect1 cell line (normal cervical epithelial cells) we used were all purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). For cell cultured, 10% FBS and 1% penicillin were added to DMEM. All the cells were cultured with 5% CO 2 and 95% air at 37 ◦ C. The culture medium was changed every 2 or 3 days according to the state of the cells. When the cells grew well, the density was between 80% and 90%.

Real-time RT-PCR analysis

Expressions of PHAP1 and SUMO2 mRNA were determined by real-time PCR, respectively. All total RNA was extracted from fresh tissues using Mini Kit (QIAGEN, Valencia, CA, USA), following manufacturer description. The total RNA concentration was measured by spectrophotometer and the purity was evaluated. RT Kit (Takara, Dalian) was used to reverse transcribe qualified total RNA into cDNA. Then, cDNA was amplified by PCR using primers specific for PHAP1 and SUMO2. The details of the reference genes used in the assays were obtained from the NCBI, and Primer Premier was applied to design and screen primers. All the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primers listed in Table 1. The 2 genes mRNA levels were calculated using the Ct method. The relative expression level of PHAP1 and SUMO2 in relation to GAPDH were performed using $2-\Delta\Delta Ct$ method.

Immunohistochemistry

All tissue samples were fixed with 10% formalin, embedded in paraffin, sectioned 4 μ m thick, immunohistochemical staining. The tissue mass is hydrated in a series of graded alcohol solutions. We used 5% hydrogen peroxide to block endogenous peroxidase, then carried out antigen repair (6 minutes × 2 times), and 6% serum was blocked for 60 minutes. The sections were then incubated with polyclonal antibody against PHAP1 and SUMO2 (1: 100 Boster Biological Technology, China) and p-AMPKa (Thr 172) (1: 100, Boster Biological Technology, China) overnight at 4°C, followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase (1: 200) at 37°C for 1 h, followed by diaminobenzidine (DAB) staining for 5–30 s.

Western blot analysis

Detection of the protein level of PHAP1 and SUMO2 in Cervical squamous cell cancer cell lines, the protein expression of PHAP1 and SUMO2 in SiHa (HPV-positive), C33A (HPV-negative) and Ect1 (normal cervical epithelial cells) cell line were detected by WB analysis. After transfection, amounts of total protein in cell lines were lysed with cold RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 72h according with the instruction for RIPA lysate. Then, we measured the protein concentration using Lowry’s method. Total proteins (50 μg per lane) were separated and then were transferred onto the PVDF membrane (BioRad, Hercules, CA, USA). The membranes were incubated at room temperature for 2h with 5% dried skimmed milk powder to block non-specific antibody binding (mouse monoclonal antibody (1:2000), HRP- Goat Anti-Rabbit Secondary Antibody (1:4000)). Finally, the protein signals were quantified and the bands were researched by using the Image Lab Software (Bio-Rad Laboratories, Hercules, CA, United States) and normalized to GAPDH.

Statistical analysis

All the statistical analyses were performed with IBM SPSS 19.0 software package (SPSS, Inc., Chicago, IL) and Graphpad Prism 5 (GraphPad Software, United States).Data are presented as mean ± SD. Group difference was performed using Student's t test. A P-value <0.05 was considered as the level of statistical significance.

Results

The PHAP1 and SUMO2 mRNA were increased in cancer tissues

To explore the potential function of PHAP1 and SUMO2, we collected fresh tissue samples, including 27(33.3%) cervical cancer cases, 35(43.2%) CIN cases,19(23.5%) normal cases. RT-PCR was performed to determine the expression of PHAP1 and SUMO2 in these fresh tissue samples. From the CSCC lesions to CIN and normal lesions, the mRNA expression of PHAP1 and SUMO2 had a downward trend, but there was no significant difference(Fig 1).

Real-time PCR analysis showed that the expression of PHAP1 was significantly higher in CSCC specimens than in CIN specimens and normal specimens. The results suggested that the PHAP1 may be a promoter instead of a inhibitor for CSCC.

Compared with the CIN group, the expression of SUMO2 mRNA was significantly up-regulated in the CSCC group, but was significantly down-regulated in the normal group. The results suggested that SUMO2 was a oncogene and plays an important role in CSCC tumorigenesis and development.

PHAP1 and SUMO2 protein were highly expression in CSCC tissues

To further explore the clinical significance of PHAP1 and SUMO2 expression in CSCC, we examined by immunohistochemistry(IHC) assay with staining on Paraffin embedded tissue (FFPE) tissues from 40 cases of CSCC,39 cases of CIN, and 23 cases of normal tissues. The representative image is shown in Fig2.

PHAP1 was dramatically increased in CSCC tissues(35/40,87.5%) compared with CIN(11/39,28.2%) tissues. On the contrary, the PHAP1 protein was negative expression in normal cervical tissues. The expression of PHAP1 is localized on the cell nucleus of tumor cells. The expression of PHAP1 was gradually increased in cervical specimens from normal tissues to CIN and CSCC. There were highly significant statistical differences between CSCC group and normal group($p<0.0001$), CSCC group and CIN group ($p<0.001$), CIN group and normal group ($p<0.0001$). The results were shown in figuer3.To further

understand the clinicopathologic significance of PHAP1 expression in CSCC, the relationship between PHAP1 expression and its clinicopathologic characteristics was analyzed (Table2). There is no significant correlations between PHAP1 expression and clinical parameters, including age(P =0.944),tumor size(P =0.247),AJCC stage(P =0.665),Infiltration degree(P =0.059),lymph node metastasis(P =0.723), differentiation(P =0.353), vascular invasion(P =0.886), nerve invasion(P =0.886).

Consistently, the expression of SUMO2 is also localized on the nucleus of tumor cells. Similar changes in SUMO2 expression were also showed. It is negative in normal cervical tissues. The positive expression of the protein was observed in 29 tumors (72.5%) and 12 CIN lesions(30.7%).Nevertheless, the expression of SUMO2 was negative in normal cervical lesions. The expression of SUMO2 was gradually increased in cervical specimens from normal tissues to CIN and CSCC. There were highly significant statistical differences between CSCC lesions and normal lesions($p<0.0001$), CSCC lesions and CIN lesions($p<0.0001$), CIN lesions and normal lesions($p<0.0001$). The results were shown in figuer3. The result showed that the high expression of SUMO2 is related to AJCC stage(p=0.024) and lymph node metastasis(p=0.020).While, there is no statistical significance between the expression of SUMO2 and age(P =0.875),tumor size(P =0.99) ,infiltration degree(P =0.633),differentiation(P =0.269), vascular invasion(P =0.267), nerve invasion(P =0.687).The results was displayed in table2.

The PHAP1 and SUMO2 expression is markedly up-regulated in CC Cell Lines and closely related to HPV16 infection

To determine whether PHAP1 and SUMO2 expression exhibited similar patterns in CC cells, we use WB (Western-blot) assay to detected the expression of the 2 proteins in SiHa(HPV16-positive), C33A (HPV16-negative) and normal cervical cells(Ect1). The relationship between the expression of these two proteins and HPV16 infection was further analyzed.

Western blot showed that the expression level of PHAP1 was significantly higher in SiHa and C33a cells than in Ect1 cells. Additionally, compared to the C33a cell line, PHAP1 protein levels were elevated in SiHa cell lines. There is statistical significance between the SiHa and Ect1 cell lines(p=0.0009) (Fig4). PHAP1 has also been suggested as a tumor promoter in CSCC. We further confirmed that the expression of PHAP1 was associated with a susceptibility to HPV16 infections.

Interestingly, the expression of SUMO2 was significantly increased in the C33a cells, compared with the Ect1 cells (P =0.012); similar significance was observed in C33a cells and Ect1 cells (P =0.0152)(Fig4). Like PHAP1,the biological role of SUMO2 as a tumor promoter in cervical cancer has been proved. Moreover, these results show that the HPV16 infections is a susceptibility factor for CC.

Discussion

The incidence of cervical cancer is increasing year by year in developed countries and developing countries, especially in underdeveloped areas. It is the second most common tumor among women worldwide, with the third highest mortality rate in the world [14]. Amounts of cases present at advanced stage due to patients are lack of specific clinical symptoms earlier. So far, there are many reports of recurrence and metastasis of CC. [15]At present, a hot topic in oncology is how to find specific molecular targets to fight against tumors. We should search for new targets which are helpful for the diagnosis and treatment of CC actively.

The PHAP1 and SUMO2 were two hot spot in CC research. By looking up the expression data of PHAP1 and SUMO2 RNAseq in the Cancer Genome Atlas(TCGA), we found that compared with the normal control group, the expression level of PHAP1 and SUMO2 in CC and other organ malignant tumors increased significantly.From the expression of PHAP1 and SUMO2 RNAseq data of (TCGA), we found that PHAP1 and SUMO2 expression levels was significantly increased in CC and other organ cancers compared with their controls (Fig. 5a Fig. 5b). Furthermore, from the Gene Expression Profiling Interactive Analysis (GEPIA), we also found that the level of PHAP1 and SUMO2 in the Cervical squamous cell carcinoma and endocervical adenocarcinoma CESC tissues (N = 306) was significantly higher than that in the controlled normal cervical tissues (N = 13) (Fig. 5c, Fig. 5d). Therefore, our research group collected fresh tissue samples and FFPE tissues of CSCC, CIN and normal speciments respectively and purchased 3 kinds of cell lines(SiHa, C33A and Ect1) aiming to investigate the ability of PHAP1 and SUMO2 gene in CSCC.

We employed RT-PCR and IHC to detect the PHAP1 and SUMO2 expression level. Our study have shown that the PHAP1 and SUMO2 mRNA and protein levels were obviously up-regulation in CSCC specimens than those in the CIN and normal specimens. Then, we used WB assay to analyze the 2 protein expression level in cell lines, including SiHa(HPV16-positive), C33A (HPV16-negative) and Ect1 (normal cervical cells). From the SiHa to C33A and Ect1 cell line, the protein expression of PHAP1 and SUMO2 had a downward trend. Interestingly, there was significant difference. All data indicated that PHAP1 and SUMO2 may accelerate the progression of CSCC and these results showed that the HPV16 infection is a susceptibility factor for CC.

Modern studies have showed that PHAP1 is a kind of fluorescent protein which have many functions. It is involved in cell differentiation, gene transcription, cell apoptosis and cell cycle transformation [16]. In the current study, PHAP1 plays pivotal roles in human cancers. Amounts of studies showed that PHAP1 as a tumor suppressor in many human cancers, including breast cancer, pancreatic cancer and non-small-cell lung cancer[17-19].However, numerous studies demonstrated that increased PHAP1 expression is correlative to hepatocellular carcinoma, colorectal cancer, prostate cancers and oral squamous cell carcinoma. These all datas suggested that PHAP1 played crucial role in the malignant phenotype regulation of cancers and can be used as a carcinogen to promote the progression of cancers [20-24]. Still, limited information about PHAP1 expression and its clinical relevance in CC patients remains unknown. In order to further explore the mechanism of PHAP1, we employed RT-PCR, IHC, WB methods to systematically explored the role of PHAP1 in the reprogramming of CC. Consistent with previous studies,

our results suggest that the expression of PHAP1 was elevated in CSCC. The PHAP1 functions as an oncoprotein in the development and progression of CSCC, which may serve as a potential biomarker for CSCC. Therefore, our results showed that aberrant expression of PHAP1 may be an important event in the development and malignant progression of CSCC.

To the best of our knowledge, the biological functions of SUMO2 in CC have little been characterized yet. According to Kunz K et al[25]. The SUMO protein family is a family in mammals which has a functional value in the regulation of many biological functions. SUMO2 is a member of the SUMO protein family.SUMO2 is a protein related to ubiquitin, which participates in the post-translational modification of lysine residues [26]. Bursomanno S et al[27],SUMO2 has been proved to be highly activated in tumor cells by binding with a number of special enzymes, which is consistent with our research. Similarly, we demonstrated that SUMO2 might be a direct effect of the CSCC.

Conclusions

We demonstrated that PHAP1 and SUMO2 were highly expressed in CSCC. Moreover, our findings also indicated that PHAP1 and SUMO2 expression were associated with disease progression in CSCC and has a strong association with HPV16 infection. Our study provide a theoretical basis that PHAP1 and SUMO2 may serve as a novel diagnosis, prognosis and treatment target. However, deeper investigations are still needed to elaborate how does PHAP1 and SUMO2 play roles in the occurrence and development of CSCC in the future study.

Abbreviations

List of Abbreviations

CC Cervical carcinoma

SCC squamous cell carcinoma

CSCC Cervical squamous cell carcinoma

CIN Cervical intraepithelial neoplasia

CESC Cervical squamous cell carcinoma and endocervical adenocarcinoma

FFPE Paraffin embedded tissue

HPV human papillomavirus

Declarations

Ethics approval and consent to participate

This study and the use of patient material in this study was conducted under protocols approved by the Medical University of Xinjiang. All experiments were performed in accordance with the relevant guidelines and regulations. All subjects provided written informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated for this study are available from the corresponding author on reasonable request.

Competing interests

No

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Authors' contributions

XHL: Collected and processed patient specimens.

SQ: Confirmed histopathological characterization of lung cancer specimens.

QL: Experimental operation, data statistics.

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Authors' information (optional)

1Department of Pathology, basic Medicine College,Medical University of Xinjiang, Urumqi, China; 2Department of Pathology, First Affiliated Hospital, Xinjiang Medical University, Urumqi, Xinjiang, China; 3Department of Gynaecology , First Affiliated Hospital, Xinjiang Medical University, Urumqi, Xinjiang, China; #correspongding author

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Tables

Table 1 List of primers used in quantitative RT-PCR analysis.

Reverse transcription-polymerase chain reaction Primer		
PHAP1	Forward: AGGATGAGGAGGAGGAAGGTGAAG Reverse: TCCTCGTCATCTACCTCTCCATCG	94bp
SUMO2	Forward: CGCTCCTGGTGCTGCTTGTG Reverse: GAGCGCCGGAGTCTCCTCAG	84bp

Table.2 Correlation between the PHAP1 and SUMO2 expression level and clinicopathological parameters in CSCC

		PHAP1				SUMO2			
Characters	No	Low	High	c^2	P	Low	High	c^2	P
Age years									
<60	33	4	29	0.05	0.944	9	24	0.025	0.875
>60	7	1	6			2	5		
Nation									
Uygur	16	1	15	0.05	0.944	6	10	0.952	0.329
Non-Uygur	24	4	20			5	19		
Tumor Size									
<3	24	3	21	1.338	0.247	6	18	0.00	0.99
>3	16	2	14			5	11		
AJCC Stage									
T0	0	0	0	0.188	0.665	0	0	7.446	0.024
T1	33	3	30			7	26		
T2	1	1	0			0	1		
T3	6	1	5			4	2		
Infiltration									
>1/2	20	3	17	5.650	0.059	6	14	0.229	0.633
<1/2	20	2	18			5	15		
Lymph									
No	34	4	30	0.125	0.723	7	27	5.431	0.020
Yes	6	1	5			4	2		
Differentiation									
Poorly	11	1	10	2.083	0.353	1	10	2.629	0.269
Moderately	21	4	17			7	14		
Well	8	0	8			3	5		
V-invasion									
No	25	2	23	0.02	0.886	7	18	1.234	0.267
Yes	15	3	12			4	11		
N-invasion									
No	31	4	27	0.02	0.886	9	22	0.162	0.687
Yes	9	1	8			2	7		

Figures

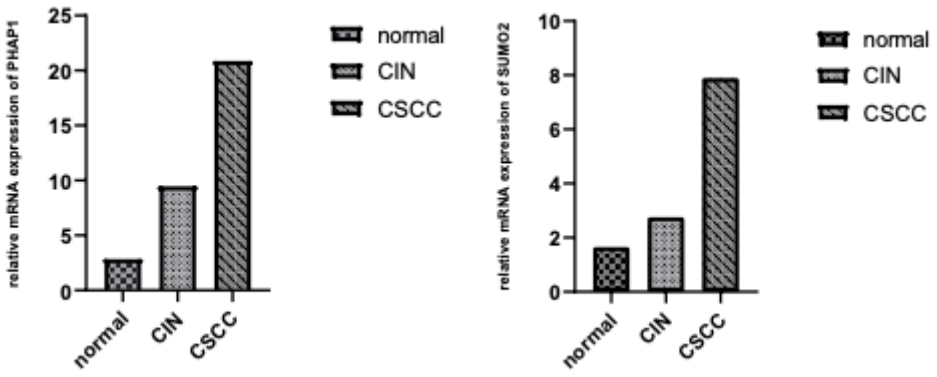

Figure 1

PHAP1 and SUMO2 is overexpressed in CSCC. RT-PCR assay were used to detect mRNA levels in CSCC tissues, CIN and normal cervical tissues.

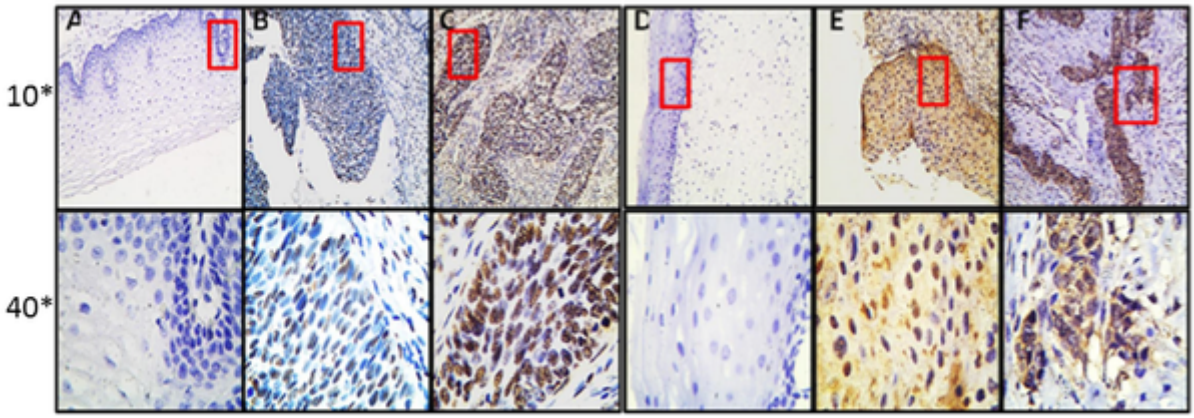

Figure 2

The expression of PHAP1 and SUMO2 in CSCC tissues, CIN tissues and normal tissues.(A) PHAP1 was negative expression in normal cervical tissue. (B) PHAP1 was weak expression in CIN tissue. (C) PHAP1 was strong expression in CCSC tissue. (D) SUMO2 was negative expression in normal cervical tissue. (E) SUMO2 was moderate expression in CIN tissue. (F) SUMO2 was strong expression in CCSC tissue.

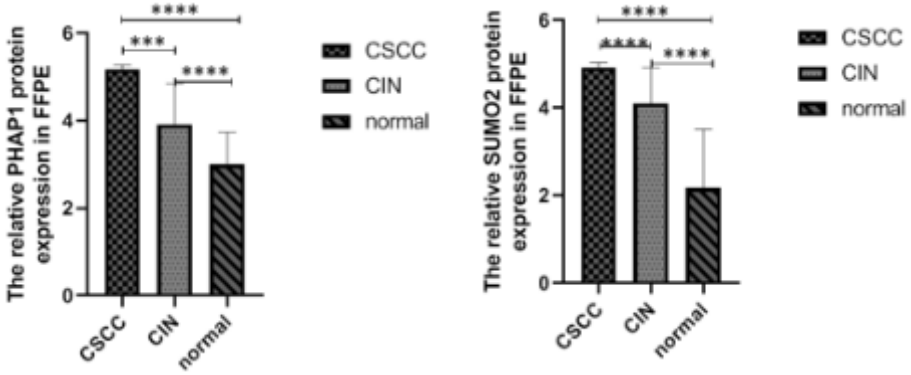

Figure 3

IHC-based analysis of protein expression for PHAP1 and SUMO2 in normal, CIN, and CCSC tissues.*statistical significance by one-way ANOVA test (****p<0.0001;***p<0.001).

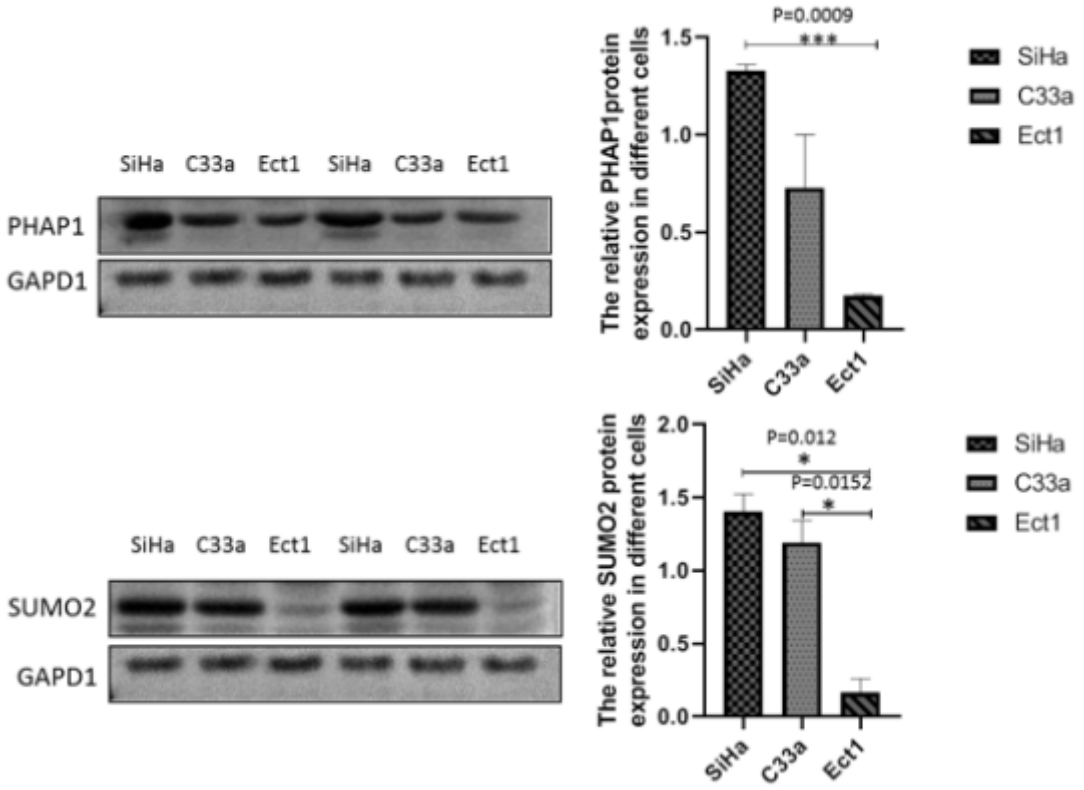

Figure 4

Western blotting was performed to detect PHAP1 and SUMO2 protein expression in C33a, SiHa, and Ect1 cells.*p<0.05,***p<0.001.

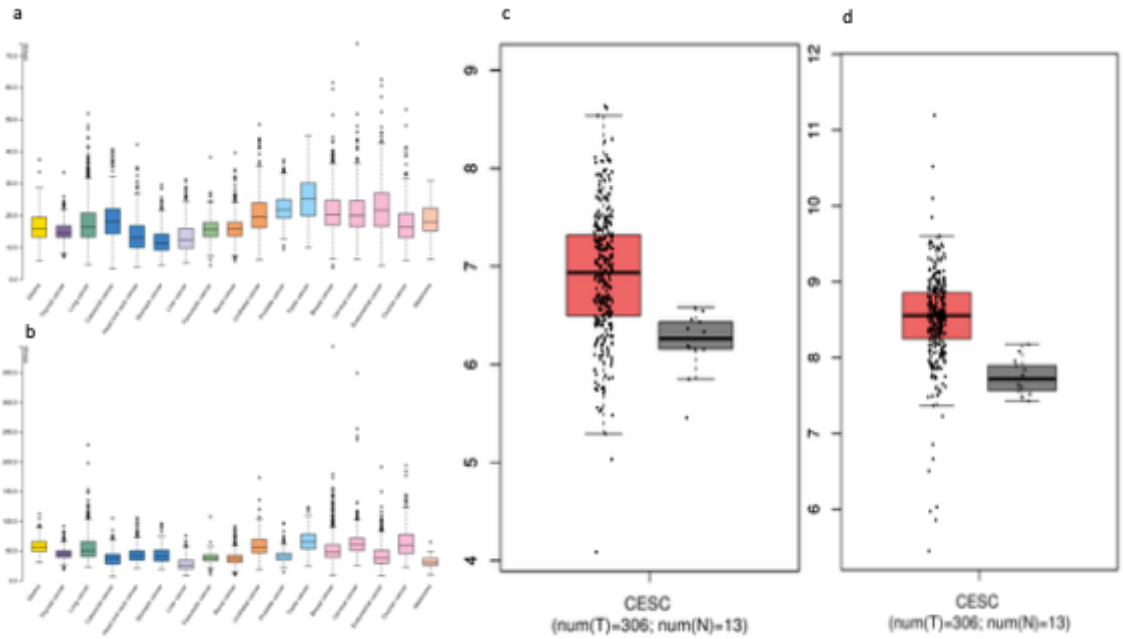

Figure 5

PHAP1 and SUMO2 were overexpressed in cervical cancer(CESC) tissues and cancer cells. a PHAP1 mRNA expression was significantly increased in cervical cancer and other organ cancers compared with their controls. b SUMO2 mRNA expression was significantly increased in cervical cancer and other organ cancers compared with their controls. c Quantification of PHAP1 mRNA expression in cervical cancer and normal tissues. d Quantification of SUMO2 mRNA expression in cervical cancer and normal tissues.