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# Overexpressed LEDGF is a novel biomarker of poor prognosis in patients with cervical cancer

**X.H. Yin, Z.Q. Wang, Q.H. Guo, H. Wu, M. Shi**

*Department of Obstetrics and Gynecology, Clinical Medical College of Yangzhou University, Yangzhou, Jiangsu (China)*

**Summary**

Lens epithelium-derived growth factor (LEDFG) can prevent cells apoptosis by activating stress proteins and anti-apoptotic protein, which are involved in the development of a variety of malignancies as some studies have shown. However, little is known about the role of LEDGF in cervical cancer. In this study, the authors collected 95 cases of the cervical cancer tissue samples and its matching tissue adjacent to carcinoma diagnosed by the Department of Pathology. mRNA expression of LEDFG in randomly selected 20 cervical cancer tissues and 20 adjacent normal tissues was detected by quantitative real-time PCR (qRT-PCR). LEDFG protein expression in randomly selected 20 cervical cancer tissues and 20 adjacent normal tissues was detected by immunohistochemistry (IHC) and Western Blot (WB). All patients were followed up for about three years. The authors found that both mRNA and protein expression level of LEDFG was significantly higher in cancer tissues compared with normal controls ($p < 0.05$) and this overexpression was significantly correlated with the histologic grade, the immersion depth of interstitial, the invasion of vessel, and lymph node status of cervical cancer. Furthermore, the three-year survival rate of 34 patients with LEDGF positive expression having a survival rate of three years was 57.6%. The survival rate of three years with negative expression was 91.7%. The survival rate of patients with LEDGF positive expression was significantly lower than those of the negative expression ($p < 0.01$). In conclusion, the present results suggest that LEDFG expression is an independent prognostic biomarker for cervical cancer.

*Key words:* Cervical cancer; LEDGF; Immunohistochemistry; Western Blot; RT-PCT.

## Introduction

Cervical carcinoma, is the second most common cancer in females worldwide. The incidence rate is just lower than breast cancer [1, 2]. There are about 500,000 new cases of cervical cancer and 275,000 die of cervical cancer each year [3, 4]. The most common pathological type is squamous cell carcinoma (SCC) in about 75% -80%; other special types include adenocarcinoma (ADC), adenosquamous carcinoma, and so on.

Currently, many studies suggest that the oncogenic types of human papillomaviruses (HPVs), such as HPV16 and HPV18, are major cervical carcinoma. Two viral oncogenes, E6 and E7, are crucial for both the induction and the maintenance of the malignant phenotype of HPV-positive cervical cancer cells, indicating that cervical cancer cells display features of a phenomenon termed ‘‘oncogene addiction’’ [5]. E6 and E7 combined with tumor suppressor protein p53 protein and phosphorylated Rb protein (pRb), respectively, induce the degradation and disturb the normal cell cycle regulation [6, 7]. However, there are few women who infected HR-HPV develop into cervical carcinoma. There must be other factors participate in the pathogenesis of cervical carcinoma.

Growing evidence supports that an augmented state of cellular oxidative stress (ASCOS) is a major contributing factor to carcinogenesis [8, 9]. Possible triggers of ASCOS include lifestyle and environmental-related factors such as diet, infections, cigarette smoking, alcohol, and pollutants [10]. ASCOS causes damage to DNA, protein, and lipids, as well as activation of stress transcription factors, leading to the activation of stress, antioxidant, inflammatory, and prosurvival pathways that contribute to malignant transformation, cell cycle deregulation, resistance to cell death and therapy, invasion, angiogenesis, and metastasis. Lens epithelium-derived growth factor p75 (LEDGF/p75) is a stress survival transcription co-activator and autoantigen which is overexpressed in various tumors. Basu *et al.* [11] analyzed the expression of LEDGF in 21 major cancer types, and revealed significantly elevated LEDGF/p75 expression in prostate, colon, thyroid, and breast cancers; however, the LEDGF expression in cervical carcinoma is still unknown.

In this study, the authors detected LEDGF expression in cervical carcinoma and the matched tumor adjacent tissue (TAT) and analyzed the correlation between the expression level of LEDGF and the clinicopathological characteristics in cervical cancer patients. They found that overexpressed LEDGF may be a possible biomarker for cervical cancer prognosis.

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## Materials and Methods

*Selection of cases*

Ninety-five patients with cervical cancer which accepted surgery therapy in the present Institute from August 2012 to June 2014 were selected in this study, with informed consent from the Subei People Hospital. The protocols used in the study were approved by the Hospital's Protection of Human Ethics Committee. No patient received preoperative radiotherapy, chemotherapy or immunotherapy. The clinical stage of cervical cancer was evaluated based on International Federation of Gynecology and Obstetrics, 2009 (FIGO 2009) criteria. Of the 95 cases, 88 were cervical carcinomas, seven cases were cervical ADC; 34 cases of Stage I and 61 cases of Stage IIa; nine with low differentiation, 41 with middle differentiation, and 45 with high differentiation; 21 with vessel infiltration and 78 without vessel infiltration; 42 with the depth of myometrial invasion < 1/2 and 53 with the depth of myometrial invasion ≥ 1/2.

*Immunohistochemistry (IHC)*

For IHC analyses, heat-induced antigen retrieval was performed by immersing the sections in a ten mM citrate buffer solution (pH 6.0) and microwaving them for 365 minutes at 550 W. Slides were cooled in the antigen retrieval solution for 20 minutes. Endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide in methanol for 20 minutes at room temperature. Sections were incubated over night at 4°C with primary antibodies diluted in PBS supplemented with 1% horse serum. The primary antibody used was mouse-anti-LEDGF. Sections were then incubated with a biotinylated secondary antimouse antibody for 30 minutes at room temperature, followed by incubation with an avidin-biotin complex peroxidase for 20 minutes at room temperature. LEDGF were visualized by a brown 3,39-diaminobenzidine and abbreviation (DAB) reaction. Sections were glass-covered and analyzed by light microscopy using a magnification up to 6400. The IHC results were independently evaluated by two pathologists based on the following criteria: (1) staining intensity: zero (negative), 1 (weakly), 2 (middle), and 3 (strongly); (2) percentage of positive cells: 1 (≤ 25%), 2 (25–50%), 3 (50–75%), and 4 (> 75%). The final IHC score was calculated by multiplying the staining intensity score by the score of positive cells.

*RT-PCR*

Total RNA was extracted using Primer 5.0. 1.5 micrograms of total RNA were used for reverse transcription using RT enzyme mix I and RT primer mix. For real-time PCR, a final reaction (20 μL) was performed using a standard protocol and the SYBR Green PCR kit on a Real-Time PCR system which was performed in triplicate, with no template controls. The $2^{-\Delta\Delta CT}$ method was used to determine relative gene expression levels with ACTB as the endogenous control to normalize the data. The primers used were as follows: human LEDGF:5'-TTCAAAGGAAGATACCGACCA-3' and5'-CTTCTGGCAGCTTTTGGAGT-3', ACTB: 5'-TGAGCGCGGCTACAGCTT-3', and 5'- TCCTTAATGTCACG-CACGATTT-3'. PCR was performed using the following cycles: 95˚C for 30 seconds, 40 cycles of 95˚C for five seconds, 60˚C for 31 seconds, and the dissociation stage: 95˚C for 15 seconds, 60˚C for one minute and 95˚C for 15 seconds.

*Western Blot (WB)*

Proteins were extracted with RIPA, and equal amounts of protein were electrophoresed on a 12% or 15% sodium dodecyl sulphate-polyacrylamide gel and subsequently transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for two hours. The membranes were incubated with the primary antibodies at 4°C overnight: mouse-anti-LEDGF and GAPDH. The membranes were then washed thrice with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for two hours. After three TBST washes, the membranes were developed using ECL plus and exposed to X-ray film. GAPDH was used as an internal loading control.

*Follow up*

All the patients were followed up to December 31, 2014. Follow-up included detailed inquiry medical history, such as postoperative vaginal bleeding, vaginal discharge, lower abdominal pain, and other clinical symptoms and pelvic dual diagnosis or three examinations, in positive side position, vaginal smears, tumor marker SCC examination, pelvic ultrasonography, and if necessary, three-dimensional CT and MRI.

*Statistical analysis*

Statistical significance of differences measured variables between the cervical cancer tissue samples and its TAT was evaluated by Chi-square test using the SPSS software version 19.0. For IHC analyses, statistical significance of differences in calculated scores between the cervical cancer tissue samples and its matching tissue adjacent to carcinoma was determined by R×C, Chi-square test using the SPSS software version 19.0. *P*-values of ≤ 0.05 were considered statistically significant.

## Results

According to the present results, the authors observed that the LEDGF was expressed in the nucleus and occasionally in the cytoplasm. In all 95 paired samples, the positive rates of LEDGF expression were 62.1% and 40.0%, respectively. Compared to TAT, the positive rate of LEDGF in cervical cancer was significantly increased ($p < 0.05$, Figure 1).

The RT-PCR result of the expression of LEDGF in cervical cancer and TAT showed that LEDGF was significantly overexpressed in cancer than in TAT at mRNA level ($p < 0.05$, Figure 2).

WB result of the expression of LEDGF in cervical cancer and TAT showed that LEDGF was significantly overexpressed in cancer tissue than in TAT at protein level ($p < 0.05$, Figure 2).

The positive rate of LEDGF with squamous carcinoma and cervical adenocarcinoma were 60.23% and 85.71%, respectively. There was no considered statistical significance between them ($\chi^2$=0.871, $p > 0.05$). The positive rate in Stages I and IIa were 67.65% and 59.02%, respectively. There was no considered statistical significance ($\chi^2 = 0.691$, $p > 0.05$). The positive rate with low, middle, and high differentiations were 100%, 58.54%, and 57.78%, respectively. There was a considered statistical significance between low and middle differentiations ($\chi^2 = 3.957$, $p < 0.05$) and there was a considered statistical significance between low and high differentiations ($\chi^2 = 4.158$, $p < 0.05$); there was however no considered statistical significance between middle and high differentiations ($\chi^2 = 40.894$, $p > 0.05$). The positive rate with and

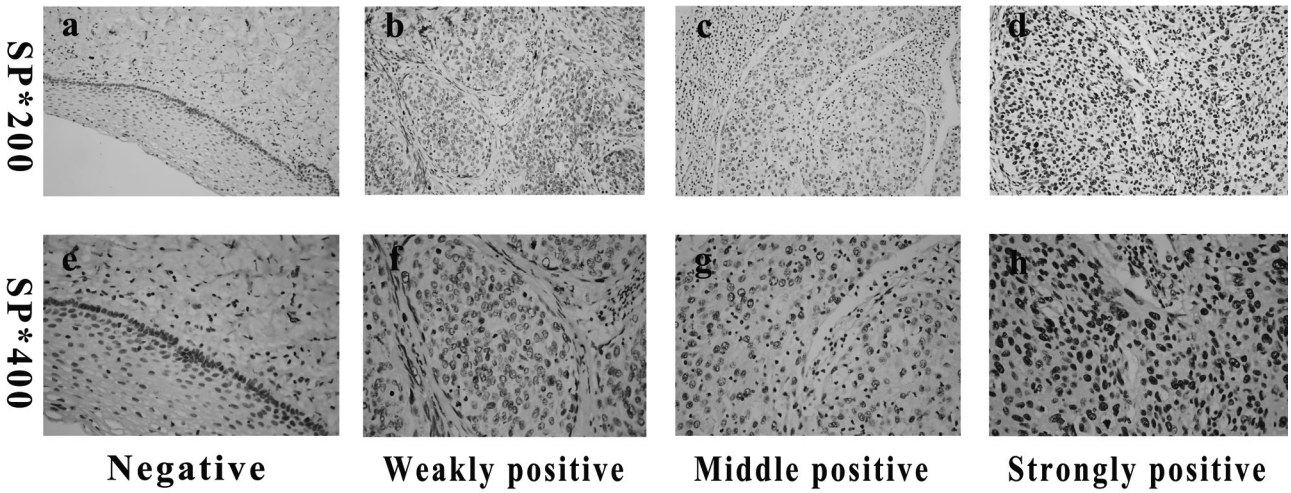


Figure 1. — LEDGF protein expression by IHC assay. (a, e) the expression of LEDGF in the cancer tissue is negative, (b, f) the expression of LEDGF in the cancer tissue is weakly positive, (c, g) the expression of LEDGF in the cancer tissue is middle positive, (d, h) the expression of LEDGF in the cancer tissue is strongly positive; a, b, c, d: SP×200; e, f, g, h: SP×400.

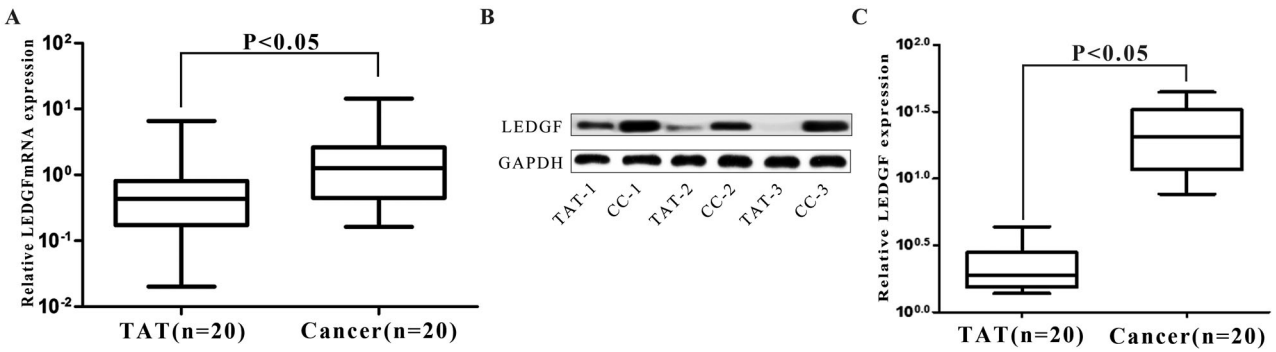


Figure 2. — The expression of LEDGF in cervical cancer and TAT. (A) LEDGF is significantly overexpressed in cancer than in TAT at mRNA level. (B, C) LEDGF is significantly overexpressed in cancer than in TAT at protein level ($p < 0.05$).

without lymphatic metastasis was 88.24% and 56.41%, respectively. There was a considered statistical significance ($\chi^2 = 6.007$, $p < 0.05$). The positive rate with and without vessel infiltration were 80.95% and 56.76%, respectively. There was a considered statistical significance ($\chi^2 = 4.069$, $p < 0.05$). The positive rate with and without deep myometrial invasion were 42.86% and 77.36%, respectively. There was a considered statistical significance ($\chi^2 = 11.851$, $p < 0.01$) (Table 1).

*Follow up and prognosis*

The authors followed up all the 95 patients; the follow-up rate was 100% and the average follow-up period was 3.5 years (from 2010 -2014 years). During the following up, 28 patients of 95 patients died, including 25 cervical cancer patients with LEDGF positive expression in 59 cases and three cases with LEDGF negative expression. The number of patients still alive was 67, with a three-year survival rate of 70.5%. Sixty-four patients had no recurrence; the recurrence rate was 32.6%. The three-year survival rate of 34 patients with LEDGF positive expression had a three-year survival rate of 57.6%. The three-year survival rate with negative expression was 91.7%.

The authors found that the survival rate of patients with LEDGF positive expression was significantly lower than those with a negative expression, and the difference between the two was statistically significant ($p < 0.01$).

## Discussion

Cervical cancer of uterus is the second common carcinomas in females and its occurrence falls behind breast cancer. Every year there are a large number of new patients with cervical cancer, and it tends to affect younger women

Table 1. — *The relationship of the LEDGF expression and the clinical date (R× C, Chi-square test).*

| Category | N | LEDGF | | Positive rate(%) | *p* |
|---|---|---|---|---|---|
| | | Positive | Negative | | |
| Pathological type | | | | | |
| SCC | 88 | 53 | 35 | 60.23 | 0.351 |
| ADC | 7 | 6 | 1 | 85.71 | |
| Differentiation | | | | | |
| Poor | 9 | 9 | 0 | 100 | 0.047[a] |
| Middle | 41 | 24 | 17 | 58.54 | 0.943[b] |
| High | 45 | 26 | 19 | 57.78 | 0.041[c] |
| Clinical Stage | | | | | |
| I | 34 | 23 | 11 | 67.65 | 0.406 |
| IIa | 61 | 36 | 25 | 59.02 | |
| Lymph node status | | | | | |
| Positive | 17 | 15 | 2 | 88.24 | 0.014 |
| Negative | 78 | 44 | 34 | 56.41 | |
| Vascular state | | | | | |
| Positive | 21 | 17 | 4 | 80.95 | 0.044 |
| Negative | 74 | 42 | 32 | 56.76 | |
| Depth of invasion | | | | | |
| < 3mm | 42 | 18 | 24 | 42.86 | 0.001 |
| ≥ 3mm | 53 | 41 | 12 | 77.36 | |

a: comparison between low and middle differentiation, $\chi^2 = 3.957$, $p < 0.05$; b: comparison between middle and high differentiation, $\chi^2 = 40.894$, $p > 0.05$; c: comparison between low and high differentiation, $\chi^2 = 4.158$, $p < 0.05$.

[12]. Studies have suggested that cervical cancer incidence rates have been increasing in recent decades in China. Systematic cervical screening with liquid-based cytology (LCT) or HPV-testing colposcopy and cervical biopsy has significantly decreased the incidence and death rate from cervical cancer. Operative treatment can be used for early-stage disease, and radiation therapy can be used for middle-late disease. Although persistent infection with HR-HPVs is the primary cause of cervical carcinogenesis, its mechanism is yet not clear.

LEDGF/p75 is a stress response protein that promotes resistance to stress-induced cell death. This protein has been implicated in inflammatory and autoimmune conditions, HIV-AIDS, and in cancer [13]. It has been studied that as a transcription co-activator, LEDGF/p75 plays an essential role in HIV-1 replication. LEDGF/p75 tethers HIV integrase to chromatin, protects it from degradation, and strongly influences the genome-wide pattern of HIV integration. Some studies revealed that as a autoantigen, LEDGF/p75 transcript expression was significantly elevated in prostate, colon, thyroid, and breast cancers [14, 15]. Studies showed that LEDGF binds to heat shock element (HSE) and stress-related regulatory element (STRE) to activate the expression of stress-related genes, such as Hsp27 and alphaB-crystallin, peroxiredoxins gene-2, ADH, ALDH, and AMPK-γ [16], which enhanced survival of many cell types. Therefore, LEDGF plays an important role in regulating cell survival and apoptosis.

*LEDGF's mechanism of molecular biology characteristics and its role in cervical cancer*

The LEDGF gene is assigned to chromosome 9q22.2 with 35740 base pairs and is adjacent to a major cell malignancy loci, which is the target site of lung cancer, leukemia, glioma, and melanoma [17]. A human LEDGF gene contains at least 15 exons and 14 introns and encodes mRNAs of LEDGF and p52; a splice variant of LEDGF. Exons 1–15 encode LEDGF mRNA, and exons 1–9 and a part of the ninth intron encode p52. Sequences of the exon/intron junctions of this gene have highly conserved sequences that abide by the GT/AG rule. LEDGF and p52 belong to a family of HDGF proteins, which include HDGF, HDGF-related protein-1, -2, and -3 (HRP-1, HRP-2, and HRP-3), LEDGF/p75, and EDGF/p52. There are 27 arginine and 84 lysine residues among 530 residues of LEDGF, which are almost the same as P75 (TCP75). It is also named LEDGF/p75. LEDGF contains several functional domains and a PWWP domain, and six potential nuclear localization signals (NLS). PWWP domain is involved in protein-DNA and protein–protein interaction [18].

Singh *et al.* [18] found that LEDGF was expressed in almost all tissues, but on specific positioning of LEDGF proteins within the cell is still controversial. Some scholars believe that it is mainly present in the nucleus [19], and it has also been reported in the cytoplasm in varying degrees of distribution [20]. In order to observe the location of LEDGF in the cell, as well as their changes with the various stages of the cell cycle, Nishizawa *et al.* [21] labeled LEDGF with green fluorescent protein (GFP), and then observed it with a fluorescence microscope. The results showed that LEDGF is secreted into the extracellular space after the turn with the cell membrane, through the membrane into the cell phagocytosis again, through the cytoplasm and the nuclear membrane, and finally gathered in the nucleus, but with no development nucleolus. Nishizawa *et al.* and Singh *et al.* [21, 22] also found that the distribution of LEDGF in the cell is also related with the temperature; when the temperature is below 28°C, the transport of LEDGF will be inhibited; at 37°C, LEDGF gathers to the nucleus; when the temperature reaches 41°C, LEDGF has gathers a high level in the nuclear membrane and cytoplasm, suggesting that external thermal stimuli may stimulate the expression of LEDGF intracellular. In this study, IHC showed that LEDGF was mainly expressed in the nucleus, appeared as brown granules, occasionally in the cytoplasm, and is more consistent with the above studies.

*LEDGF expression and clinical significance in cervical cancer*

Previous studies have confirmed that HPV is the most important factor for cervical cancer. HPV oncoprotein can silence tumor suppressor gene, especially E6 and E7 proteins. The identification of cellular targets attacked by the HPV oncogenes is critical for our understanding of the molecular mechanisms of HPV-associated carcinogenesis and

may open novel therapeutic opportunities. In order to search for cellular genes targeted by the viral E6/E7 oncogenes, Kuner *et al.* [23] silenced endogenous HPV18 E6/E7 expression in HeLa cervical carcinoma cells by RNA interference (RNAi) and performed a genome-wide transcriptome analysis. Data from this array suggested that the expression of the LEDGF gene (alternatively called PSIP1) is reduced upon E6/E7 repression.

Leitz *et al.* [24] showed that compared with normal cervical tissue, the expression levels of LEDGF were significantly increased in cervical lesions and cancer, and the expression levels were correlated with the severity of disease. This experiment compared the expression in LEDGF in 95 cases of cervical carcinoma and matched adjacent tissues by IHC and found the positive rate TO BE 62.1% in cervical cancer, while 40% in the adjacent pair tissues, showing a significant difference ($P < 0.01$). Furthered RT-PCR and WB experiments explored the expression of LEDGF in the levels of mRNA and protein, and the results strongly suggested that there was a significantly higher expression level of LEDGF in cervical cancer than in adjacent tissues ($p < 0.05$), and this is consistent with the above-mentioned literature.

LEDGF is crucial for the protection of tumor cells against various forms of cellular stress, including DNA damage. LEDGF also can more directly promote tumor progression and metastasis. As reported, LEDGF can mediate vascular endothelial growth factor activation–C, directly promoting tumor angiogenesis in glioma, lung, and ovarian cancer model [25]. The present study compared the relationship between the expression levels of LEDGF in cervical cancer and clinico-pathological parameters, analyzed the relationship between LEDGF expression levels and tumor type, clinical stage, degree of differentiation, lymph node metastasis, in the presence or absence of vascular invasion, and between stromal invasion depth. The results showed no significant difference in the expression levels of LEDGF in cervical SCC and ADC of the cervix, and with the clinical stage, and with no correlation; however, LEDGF expression levels depended on the degree of tumor differentiation: compared to the well- and moderately-differentiated groups, LEDGF was increased in poorly-differentiated group and the difference was statistically significant ($p < 0.05$), but the well-differentiated group showed no significant difference ($p > 0.05$). In addition, LEDGF according to lymph node metastasis, vascular invasion, and cervical stroma infiltration also showed no significant difference ($p < 0.05$). Histological grade, depth of stromal invasion, whether the invasion and lymph node metastasis is an important indicator to assess the degree of malignancy within the vessel, are also important prognostic factors in patients, and it can be concluded that LEDGF is involved in the invasion and cervical cancer transfer process, and it could indicate the severity of the cancer patient's prognosis. In cancer tissues, LEDGF and VEGF-C coactivator allow people to pathophysiology of the disease have a deeper understanding, but the specific mechanism of tumor cells upregulated LEDGF is unclear, there are to be further studied.

*LEDGF expression in other malignant tissues*

Basu *et al.* [26] researched the expression of LEDGF in prostate cancer. Firstly, they demonstrated that LEDGF/p75 is an autoantigen in PCA that is overexpressed in prostate tumor cells and tissues, then they used qPCR array to identify genes exhibiting significant expression changes in response to knockdown or overexpression of LEDGF/p75 in PC-3 cells. Finally, cytoglobin (CYGB), phosphoinositide-binding protein PIP3-E/IPCEF-1, superoxidase dismutase 3 (SOD3), thyroid peroxidase (TPO), and albumin (ALB) exhibited significant transcript down- and up-regulation in response to LEDGF/p75 knockdown and overexpression, respectively.

In 2009, seeking for the molecular mechanism, Sapoznik *et al.* [27] examined the role of LEDGF/p75 and the possible contribution of its putative target; a conserved stress response element (STRE) was identified in silico in the VEGF-C promoter. Using chromatin immunoprecipitation it was shown that LEDGF/p75 indeed bound the VEGF-C promoter, and this binding was augmented by FSH.

## Conclusion

Currently, understanding the mechanism of LEDGF in human tumors is in the preliminary study stage, and the present study also confirmed that LEDGF expression in only matched adjacent tissues was significantly higher in cervical cancer. LEDGF implications in the role in the development of cervical cancer mechanism, still requires research of the following: LEDGF specific mechanisms of interaction with HPV, LEDGF, and DNA biological effects combining to produce precise signal transduction pathways, LEDGF inducing tumor angiogenesis exact mechanism, and LEDGF in the regulation of cell proliferation and apoptosis. A prospective study of specific mechanisms is still required in larger samples. With further future research in these areas, LEDGF can possibly lead to elucidating the mechanism of the development of cervical cancer and create a new field in its diagnosis and in the evaluation of the prognosis, while becoming an effective indicator of cervical cancer and providing potential new treatment targets.

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Corresponding Author:
X.H. YIN, M.D.
Department of Obstetrics and Gynecology
Clinical Medical College of Yangzhou University
98 W Nantong Rd, Yangzhou
Jiangsu 225001 (China)
e-mail: yinxh1965@126.com