JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2023, 74, 1, 77-83
www.jpp.krakow.pl | DOI: 10.26402/jpp.2023.1.08

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THE ROLE OF INTERFERON-STIMULATED GENE 15 IN THE OCCURRENCE AND PROGRESSION OF CERVICAL SQUAMOUS CELL CARCINOMA

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To identify molecular markers for early diagnosis and new targets for treatment of cervical squamous cell carcinoma. Our study involved 52 carcinoma tissues that were confirmed pathologically as cervical squamous cell carcinoma (CSCC) at the Fourth Hospital of Hebei Medical University in 2021. We obtained 36 control specimens from patients who had undergone hysterectomy for benign uterine diseases in 2021, with no cervical lesions as confirmed by pathology. Total RNA was extracted from all the samples. Reverse transcription and quantitative real-time PCR were performed. Immunohistochemical staining for interferon-stimulated gene 15 (ISG15) protein was performed. Descriptive analyses including mean and standard deviation were used to compare different groups. For data that do not conform to normal distribution, we use Wilcox rank sum test to make statistics to compare different groups with the median and interquartile. Mann Whitney U test was used to compare non-parametric continuous data, and categorical variables were analyzed using chi-square test. Receiver operating characteristic (ROC) curve was used to evaluate the possibility of using ISG15 as a new biomarker for cervical squamous cell carcinoma. Compared with normal cervical tissues, mRNA expression of ISG15 in cervical cancer tissues was significantly lower ($P<0.01$); mRNA expression was significantly lower in patients with nerve invasion ($P<0.05$). Difference in ISG15 protein expression was statistically significant (no expression/low expression) in the cancer samples compared to normal tissues ($P<0.01$). The area under ROC curve was 0.810 ($P<0.001$) and the sensitivity and specificity were 75% and 54%, respectively. Spearman's correlation analysis showed that ISG15 mRNA was positively correlated with protein expression ($r=0.358$, $P=0.001$). Deficiency of ISG15 may be associated with the occurrence and progression of CSCC. It could be used as a potential tumor marker in research and treatment of CSCC.

Key words: *cervical cancer, cervical squamous cell carcinoma, interferon-stimulated gene 15, cancer occurrence and progression, metastasis, gene expression, molecular markers*

INTRODUCTION

Cervical cancer is one of the most common gynecological malignancies with 604,000 new cases and 342,000 deaths in a year, and was the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women worldwide in 2020 (1). With the emergence of earlier sexual activity and multiple sexual partners, the incidence of cervical cancer has shown an increasing trend in younger age groups, and has a serious impact on women's physical and mental health (2). Although the 5-year overall survival rates for early-stage cervical cancer was 97%, it was as low as 32% and 9% for stages III and IV, respectively, among cervical cancer patients (3-4). Studies have confirmed that the occurrence of cervical cancer was related to human papillomavirus (HPV) infection (5), but the molecular mechanism of its occurrence and progression have not been fully clarified. This requires extensive study of the molecular mechanism of the occurrence and progression of cervical cancer, to find molecular markers for its early diagnosis and new targets for treatment, while providing a new theoretical and practical basis for the prevention and treatment of cervical cancer.

Interferon-stimulated gene 15 (ISG15) encodes a 15Kd protein that can be detected as either free ISG15 or covalently associated with its target proteins through a process termed ISGylation. ISG15 is a small protein with implications in some biological processes and pathologies that include many cancers (6). For example, Zhao *et al*. found that ISG15 was involved in the progression of endometrial carcinoma (7). But there are not many studies of ISG15 reported in cervical cancer. In this study, we detected the expression of ISG15 in human cervical squamous cell carcinoma tissues to understand the role of ISG15 in the occurrence and progression of cervical squamous cell carcinoma.

MATERIALS AND METHODS

Human cervical cancer tissue specimens

This study was approved by the Institutional Human Ethics Committee of the Fourth Hospital of Hebei Medical University (2020KY266), and prior informed consent was obtained from all patients. We included 52 case group specimens and these were confirmed pathologically to be cervical squamous cell carcinoma at the Fourth Hospital of Hebei Medical University. We obtained 36 control specimens from patients who had undergone hysterectomy for benign uterine diseases and had no cervical lesions as confirmed by pathology. The 52 patients from whom the samples were obtained had undergone radical hysterectomy without previous radiotherapy and chemotherapy in 2021. The patients ranged in age from 20 to 73 years (mean age, 53 years).

RNA extraction and quantitative real-time reverse transcription PCR (RT-qPCR)

Total RNA was extracted from the samples using the RNAeasy™ Animal RNA Isolation Kit with Spin Column (Beyotime Co. Ltd., Shanghai, China). The RNA concentration and purity were measured with the Nano Drop ND-2000 spectrophotometer (ThermoFisher, Waltham, MA, USA). Reverse transcription and quantitative real-time PCR were performed using a Thermo Scientific RevertAid RT kit (ThermoFisher, Waltham, MA, USA) and BeyoFast™ SYBR Green qPCR Mix (2X) (Beyotime Co. Ltd., Shanghai, China) according to the manufacturer's protocol. All experiments were repeated thrice. GAPDH was considered as the internal reference. Relative level of ISG15 was calculated using the $2^{-\Delta\Delta Ct}$ method. The sequence of the forward and reverse primer for ISG15 and GAPDH used in the study is shown in *Table 1*. The ISG15 PCR product length was 182bp.

Immunohistochemical (IHC) assay

IHC staining for ISG15 protein was performed on 4 µm-thick sections. Tissue sections were dewaxed in xylene and in dehydrated graded ethanol. After blocking endogenous peroxidase activity and non-specific antibody binding, sections were incubated with primary antibody (rabbit polyclonal anti-ISG15 antibody, Affinity, DF6 316# #536; dilution 1:50) and left overnight at 4°C. Slides were then incubated with the secondary antibody (biotin-labeled Goat anti-rabbit IgG) and the third antibody (horseradish labeled streptomycin working solution) for 30 minutes at 37°C, respectively. After washing the slides in phosphate buffered saline (PBS), they were incubated in DAB (3,3'-Diaminobenzidine) (brown) and counterstained with hematoxylin (blue). IHC staining was evaluated by a scoring method that was previously reported (8). The score was established corresponding to the sum of: 1) the percentage of positive cells (0: 0% positive cells; 1: <25% positive cells; 2: 26–50% positive cells; 3: >50% positive cells); and 2) the staining intensity (0: negative; 1: weak; 2: moderate; 3: high). The sum for the assigned values (the positive cell percentage and the staining intensity) was 6 or <6. Scores between 0–2 was regarded as negative, 3–4 as weakly positive, and 5–6 as strongly positive.

Statistical analysis

All the experiments were repeated thrice. We used SPSS 21.0 software for statistical analysis of the data. Descriptive analyses including mean and standard deviation, for data that do not conform to normal distribution, we use Wilcox rank sum test to make statistics to compare different groups with the median and interquartile. Mann-Whitney U test was used to compare non-parametric continuous data, and categorical variables were analyzed using chi-square test. Receiver operating characteristic (ROC) curve was used to evaluate the possibility of using ISG15 as a new biomarker for cervical squamous cell carcinoma. Spearman's correlation analysis was used to evaluate the association between ISG15 mRNA expression and ISG15 protein expression. $P<0.05$ was considered statistically significant.

RESULTS

We measured mRNA expression of ISG15 in 52 human cervical squamous cell carcinoma tissues and 36 normal cervical tissues using RT-qPCR. The expression was low in human cervical squamous cell carcinoma tissue specimens. With the expression of normal tissue as a standard 160.91±0.55, the relative level was 42±0.27 in the cancer tissues ($P<0.01$), (*Fig. 1*).

Our IHC results showed that the ISG15 protein had low levels of expression in the human cervical squamous cell carcinoma tissue samples when compared with levels in the normal samples (52 vs. 36 samples, respectively). Difference in ISG15 expression (no expression/low expression) between the cancer samples and the normal tissues was statistically significant ($P<0.01$), (*Table 2*, *Fig. 2*).

Spearman's correlation analysis showed that mRNA expression of ISG15 positively correlated with ISG15 protein expression, determined by IHC score analysis ($r=0.358$, $P=0.001$), (*Fig. 3*).

In an attempt to obtain the information about the potential prognostic value of ISG15 determination and its involvement in processes of neoplastic invasion we correlated mRNA ISG15 expression, found in cervical cancerous samples, with pathohistological characteristics of the carcinoma and clinical finding in the patients (serum range of squamous carcinoma cell (SCC) marker, pathological grade of carcinoma and presence or absence of local, lymphonodal and perineural infiltration). As shown in *Table 3*, we found significantly lower levels of mRNA ISG15 only in patients with perineural infiltration ($p=0.019$), indicating that deficiency of ISG15 might be linked with neoplastic invasion of nerves and metastatic spread of cervical carcinoma.

Table 1. Primer sequences of ISG15 gene and GAPDH.

Primer	**Primer sequence**	**PCR product length**
ISG15	forward CAGATCACCCAGAAGATCGG	182bp
	reverse CCCTTGTTATTCCTCACCAGG	
GADPH	forward ACCACAGTCCATGCCATCAC	
	reverse TCCACCACCCTGTTGCTGTA	

Since according to our data ISG15 might affect both local and metastatic carcinogenic processes we plotted also ROC curve. The final result was that the area under ROC curve was 0.810 (P<0.001) and the sensitivity and specificity were 75% and 54%, respectively. This demonstrated that ISG15 could be used as a potential tumor marker in research and clinical use pertaining to cervical squamous cell carcinoma (*Fig. 4*).

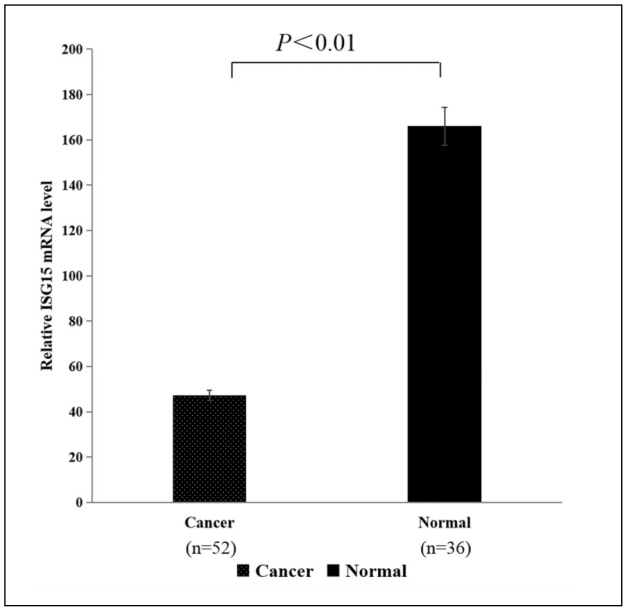

Fig. 1. mRNA expression levels of ISG15 in cervical squamous cell carcinoma and normal tissues were detected by qRT-PCR (*P<0.01).

DISCUSSION

In the United States, the potential benefits of the HPV vaccine are so promising that almost eliminating cervical cancer is considered an achievable goal (9, 10). But the molecular mechanisms involved in the progression of cervical cancer remain unclear. Thus, it is necessary to further explore these molecular mechanisms and identify reliable tumor markers. In the present study, we found that ISG15 was expressed in low levels in human cervical squamous cell carcinoma tissues. The low expression of ISG15 was statistically associated with metastasis of cervical squamous cell carcinoma, which indicated that ISG15 could be used as a potential tumor marker in research and clinical use pertaining to cervical squamous cell carcinoma.

ISG15 is a 15 kDa ubiquitin-like protein that can be secreted to the extracellular medium (11). There is increasing evidence emerging that ISG15 is implicated in a variety of pivotal cellular processes, such as DNA repair, autophagy or protein translation, as well as the development of cancer (11). However, ISG15 plays a strikingly ambiguous role in cancers. Chen *et al*. (12) reported that ISG15 expressed in nasopharyngeal carcinoma (NPC) cells were related to poor prognosis in patients with NPC. Kariri *et al*. (13) pointed out that high levels of ISG15 were associated with poor outcomes among patients with invasive breast cancer. Li *et al*. (14) investigated the function of ISG15 in hepatocellular carcinoma (HCC) progression. Their results indicated that ISG15 was highly expressed in HCC tissues and cell lines, which could

Table 2. Expression of ISG15 protein in cervical squamous cell carcinoma tissues and normal tissues was detected by IHC.

Group	**No/Low-expression**	**High-expression**	χ^2	***P***
Case	40	12	18.769	<0.001
Control	11	25		

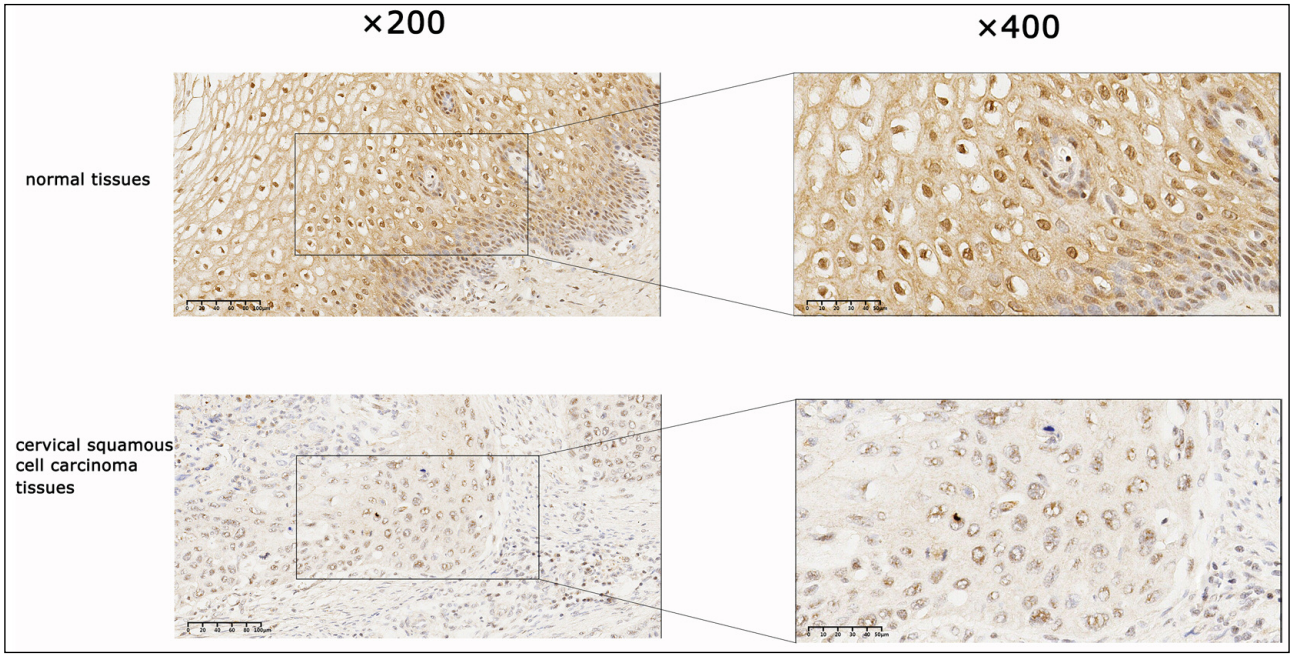

Fig. 2. Expression of ISG15 in cervical squamous cell carcinoma tissues and normal tissues, as detected by immunohistochemical assay (IHC).

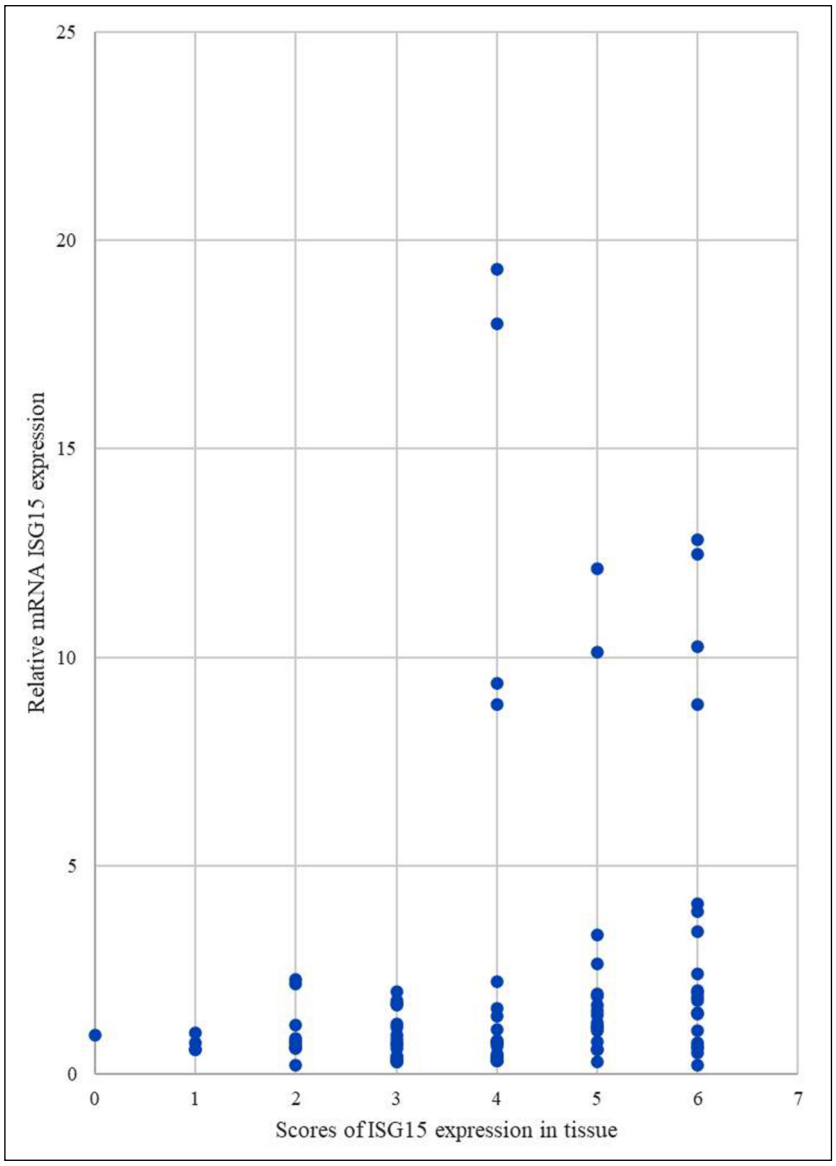

Fig. 3. Correlation analysis between the mRNA and protein expression of ISG15. The correlation analysis between the mRNA and protein expression of ISG15 are in both squamous cell carcinoma and normal cervix tissue samples (r=0.358, P=0.001).

promote the proliferation and migration of HCC cells (14). High ISG15 expression was also observed in patients with endometrial carcinoma (EC), which was associated with poor clinical outcomes and pathological stage (7). Data from the above studies suggest that ISG15 may have a tumor-promoting role in several cancers, and could be a promising marker for cancer diagnosis and prognosis. However, several published data also demonstrated that ISG15 exhibited an anti-cancer role in some types of cancers. Using cervical cancer cells, Zhou *et al*. (15) found that ISG15 could inhibit cancer cell growth and promote apoptosis. In a study, Zhang *et al*. found that low ISG15 expression was associated with poor prognosis in patients with ovarian cancer (16). Downregulating ISG15 expression may be considered as a new therapeutic strategy in the treatment of cisplatin-resistant ovarian cancer (16). In Mao's study,18 ISG15 was found to induce cancer cell apoptosis, playing an important role in tumor suppression in cervical cancer models. Thus, ISG15 may play different roles in different types of cancers.

Consistent with the findings of Zhang *et al*. (16) and Mao (17), we also found that low expression of ISG15 was related to the occurrence of cervical squamous cell carcinoma. In this study, we measured the mRNA expression of ISG15 in 52 human cervical squamous cell carcinoma tissues using RT-qPCR. Our results showed that ISG15 was expressed in significantly low levels in human cervical squamous cell carcinoma tissue specimens. We further examined the IHC of ISG15 protein in the tissues of the cervical squamous cell carcinoma samples. ISG15 was also expressed in significantly low levels in the cancer samples showing weak immunoreactivity to human ISG15 Ab ($P<0.01$). The above data confirmed that the low expression of ISG15 could be related to the occurrence of cervical squamous cell carcinoma.

Using the ROC curve analysis, we evaluated the possibility of using ISG15 as a new biomarker for cervical squamous cell carcinoma. The area under the ROC curve (AUC) was 0.810, which indicated that ISG15 could be a tumor marker of cervical squamous cell carcinoma. We further analyzed the correlation between ISG15 and clinical characteristics of patients with cervical squamous cell carcinoma, such as, SCC, pathological grade, neural invasion, and FIGO stages. Neural invasion is the fifth most frequent route of cancer spread, and is different from transcoelomic, lymphatic spread, hematogenous spread, and canalicular spread (18). It has been found that cervical cancer exhibits a tendency toward neural invasion (18). Neural invasion

Table 3. Association of the ISG15 mRNA with pathohistological characteristics of cervical squamous cell carcinoma and clinical findings in patients.

Factor	No.	mRNA($2^{-\Delta\Delta Ct}$)	P
Serum levels of squamous carcinoma cell marker (ng/ml)			0.187
<2.7	20	1.04±0.61	
≥2.7	32	0.83±0.50	
Pathological grade			0.179
Keratinizing type	28	0.81±0.55	
Non-keratinizing type	24	1.02±0.54	
Lymph node infiltration			0.378
No	34	0.86±0.54	
Yes	18	1.00±0.58	
Perineural infiltration			**0.019**
No	46	0.96±0.56	
Yes	6	0.48±0.12	
Invasion of the internal opening of cervical canal			0.825
No	34	0.85±0.45	
Yes	18	1.02±0.70	
Invasion of the uterine cavity			0.998
No	48	0.91±0.57	
Yes	4	0.91±0.16	
FIGO stage			0.813
I	26	0.86±0.56	
II	6	0.91±0.58	
III	20	0.97±0.56	

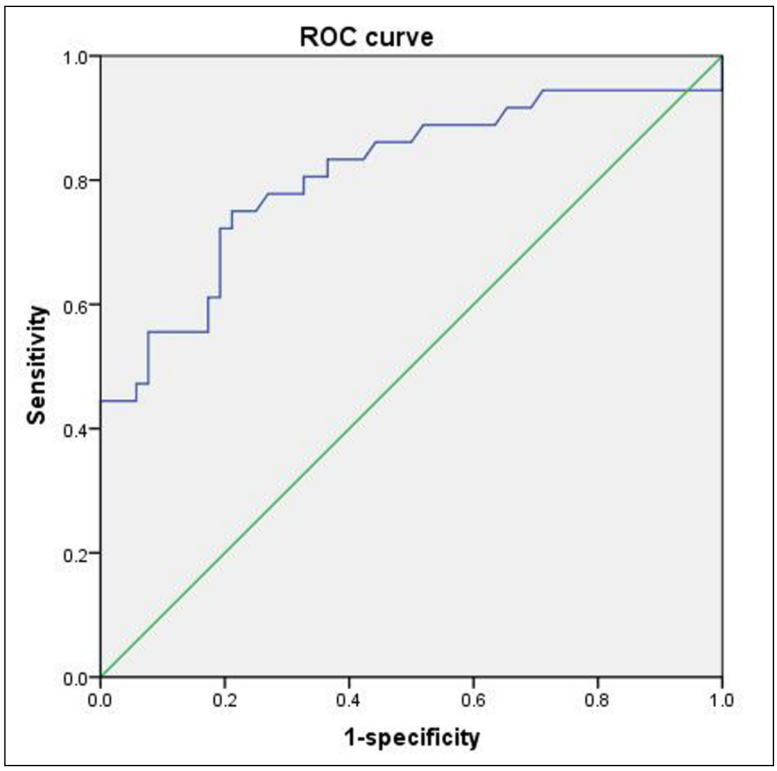

Fig. 4. Evaluation of ISG15 expression in cervical squamous cell carcinoma tissues, as analyzed using ROC curve.

was reported to be significantly associated with multiple high risk and intermediate-risk factors for recurrence (18). In this study, we found that the mRNA expression was significantly lower in patients with nerve invasion, which suggests that ISG15 may be associated with metastasis of cervical squamous cell carcinoma. This has a certain significance in highlighting the clinical characteristics of ISG15 and the progression of cervical squamous cell carcinoma.

The occurrence and development of cervical cancer may not be caused by a single factor. It was the result of a comprehensive impact of multiple factors. Recent research showed hydroxycarboxylic acid receptor 1 (HCAR1/GPR81) plays a vital role in cancer biology, HCAR1 in development of chemoresistance in cervical carcinoma HeLa cells *via* ABCB1 transporter up-regulation which might be implicated in the cervical cancers *in vivo* (19). Similar to ISG15 in our current study, it may shed light on the formation factors of cervical cancer. Wang *et al*. (20). showed that the downregulation of LINCO1554 gene might be responsible for malignant progression and the anti-cancer drugs resistance observed in laryngeal squamous cell carcinoma. These two articles have analyzed the mechanism of occurrence, but our research tends to highlight biomarkers of cervical cancer. Therefore, we focused on studying the expression of ISG15 and its identification efficiency in biology. Therefore, we conducted ROC curve analysis to ultimately confirm that ISG15 may become an effective marker. Our results also explored the role of genetic factors and aberrations in the occurence and progression of cervical squamous cell carcinoma. As these additional relatively novel evidences but also clinically-relevant observations will allow the researchers of physiology and pharmacology gain a better mechanistic insights into the pathomechanism of the cervical tumorigenesis with some elements to practical understanding of the potential tumor markers as well as targets of pharmacotherapy that has been currently taylored in human subjects with cervical cancer.

Our study has some limitations. The sample size of this study was small, with only 52 cases undergoing RT-qPCR and IHC. The research methodology was simple. However, we are currently collecting more specimens to expand the sample size and conduct further *in vitro* and *in vivo* experiments to uncover more relevant mechanisms of ISG15 involvement in the occurrence and progression of cervical squamous cell carcinoma.

In conclusion, our study confirmed that low expression of ISG15 in cervical squamous cell carcinoma tissues could play a key role in carcinogenesis of cervical squamous cell carcinoma. The findings suggest that ISG15 could be a potential molecular target in cervical squamous cell carcinoma.

Funding: This study was supported by Hebei Province Medical Science Research Youth Science and Technology Project(number 20210334).

Availability of data and materials: All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Acknowledgements: We would like to acknowledge the hard and dedicated work of all the staff that implemented the intervention and evaluation components of the study.

Conflict of interests: None declared.

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Received: January 16, 2023
Accepted: February 28, 2023

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