Interferon-stimulated gene 15 promotes progression of endometrial carcinoma and weakens antitumor immune response

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Abstract. Endometrial carcinoma (EC) is one of the most common gynecological cancers with a poor prognosis. Therefore, clarifying the details of the molecular mechanisms is of great importance for EC diagnosis and clinical management. Interferon-stimulated gene 15 (ISG15) plays an important role in the development of various cancers. However, its role in EC remains unclear. High ISG15 expression was observed in EC, which was associated with poor clinical outcomes and pathological stage of patients with EC, thus representing a promising marker for EC progression. Further exploratory analysis revealed that the elevated ISG15 levels in EC were driven by aberrant DNA methylation, independent of copy number variation and specific transcription factor aberrations. Accordingly, knockdown of ISG15 by small interfering RNA attenuated the malignant cellular phenotype of EC cell lines, including proliferation and colony formation *in vitro*. Finally, investigation of the molecular mechanisms indicated that ISG15 promoted the cell cycle G1/S transition in EC. Furthermore, ISG15 promoted EC progression by activating the MYC proto-oncogene protein signaling pathway. Moreover, ECs with high levels of ISG15 harbored a more vital immune escape ability, evidenced not only by significantly less invasive $CD8^+$ T cells, but also higher expression of T cell inhibitory factors, such as programmed death-ligand 1. These results suggest a tumor-promoting role of ISG15 in EC, which may be a promising marker for diagnosis, prognosis and therapeutic immunity.

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Introduction

Endometrial carcinoma (EC) is the sixth leading cause of cancer-related deaths among women, resulting in an estimated 11,350 deaths annually (1). Despite effective control of the overall incidence of cancer, the number of newly diagnosed ECs has increased steadily over the years (2). Although a number of patients are diagnosed with early-stage disease, 15-20% of patients with EC experience recurrence after primary surgery for metastatic disease. Thus, more effective biomarkers and targets for EC diagnosis or treatment strategies are urgently needed (3-5).

Post-translational modifications (PTMs) have critical roles in cancer progression, among which ubiquitin and ubiquitin-like (UBL) protein modifications are the most abundant PTMs that determine protein fate (6,7). Recently, the UBL system has received increasing attention, but breakthroughs are still lacking (8-10). A panel of UBL proteins has been identified, including small UBL modifiers (11), neural precursor cell expressed, developmentally down-regulated 8 (12), autophagy-related protein (ATG)8 and ATG12, and interferon-stimulated gene 15 (ISG15) (13,14). ISG15 is a 15 kDa protein that reportedly regulates antiviral defense and provides immunomodulation (15,16). Aberrant ISG15 expression has been reported in various types of cancer, including nasopharyngeal carcinoma, hepatocellular carcinoma and breast cancer (17-19).

Activation of the type I interferon (IFN) signaling pathway is a key component of anticancer innate immunity (20). The transcription of ISG15 is induced by type I IFN signals and IFN-induced enhancer elements mediate the effect of IFN-α on ISG15 transcription (21). ISG15 expression is related to the induction of IFN and the activation of immune cells, both of which are important mediators of tumor immunity (22). Previous studies showed that melanoma cells secrete free ISG15 to regulate the phenotype of tumor-infiltrating dendritic cells (DCs) (23,24). High ISG15 levels have also been detected in the culture medium of melanoma, along with the strong positive expression of ISG15 in the cytoplasm of melanoma specimens (25). Thus, ISG15 plays a tumor-promoting role in tumor immunogenicity. When ISG15 is secreted, free ISG15 can act as a cytokine to regulate the immune response (26). Moreover, free ISG15 can activate natural killer cells, enhance

lymphokine-activated killer-like activity, stimulate IFN-γ production, induce DC maturation and recruit neutrophils (27).

The expression pattern, biological functions and underlying mechanisms of ISG15 in EC remain unclear. In this study, the expression levels of ISG15 in EC and the relationship of ISG15 with clinicopathological features and patient survival were investigated. Furthermore, the potential molecular mechanism of ISG15 in promoting tumorigenesis was also explored.

Materials and methods

Samples. All ISG15 expression data in clinical samples, including both RNA and protein levels, were derived from the bioinformatics analysis. Cell-related functional and mechanistic experiments, including polymerase chain reaction (PCR), western blotting and flow cytometry, were performed at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). This study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University (approval no. 2020KY065). All patients who contributed to the research provided their written consent.

Gene expression analysis in tumor samples from the public database. Differential RNA expression data between tumor and normal tissues collected from The Cancer Genome Atlas (TCGA) database were downloaded (28) and analyzed using the online tools MEXPRESS (https://mexpress.be/) and Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) (29). Transcription factor analysis against ISG15 was performed online using the Cistrome website (http://cistrome.org/db); the purpose of this database is to use ChIP sequence data to analyze the lacation of binding sites of transcription throughout the genome *in vivo* (30). The proteomic data for EC were obtained from the online Uniprot website (http://www.uniprot.org/). Protein IHC analysis, mRNA (FPKM) and corresponding clinical information data were downloaded from The Human Protein Atlas (https://www.proteinatlas.org/).

Immunohistochemistry (IHC). Tissue array with 11 normal endometrial and 34 EC tissues were provided by National Human Genetic Resources Sharing Service Platform (China; HUteA045PG01). Written informed consent was obtained from the patients undergoing surgery. The clinical characteristics of the patients are described in Table SI. The tissue was fixed with 4% paraformaldehyde at room temperature for 72 h, then embedded in paraffin and cut into 4-μm serial sections for immunohistochemical study. For IHC analysis, the tissue sections were deparaffinized with xylene at room temperature and rehydrated using a gradient ethanol solution. Endogenous catalase activity was blocked using 3% hydrogen peroxide for 10 min in the dark at room temperature. Tissue sections underwent antigen retrieval in 0.01 M sodium citrate buffer for 5 min with a pressure cooker and were cooled at room temperature. Primary antibodies against ISG15 (catalog no. ab285367; Abcam) were diluted in phosphate-buffered saline (PBS) at 1:200 and incubated with the slices overnight at 4˚C. The reactions were visualized according to the manufacturer's instructions using the Enhanced Polymer DAB detection kit (OriGene Technologies, Inc.) and an optical microscope.

Tumor IMmune Estimation Resource (TIMER) analysis. TIMER2.0 (http://timer.cistrome.org/) provides a more robust estimation of immune infiltration levels for TCGA or user-provided tumor profiles using six state-of-the-art algorithms (31). TIMER was used in this study to evaluate the correlation between the expression of ISG15 and the level of immune infiltration. The somatic copy number alteration (SCNA) module in the TIMER database can relate the rate of tumor infiltration of a gene to various changes in somatic copy number. SCNA incorporates high amplification (2), arm-level gain (1), normal diploid (0), arm-level deletion (-1) and deep deletion (-2). For each SCNA group, the degree of infiltration was compared to the average using a two-sided Wilcoxon test.

Cell culture. Human EC HEC-1A and Ishikawa cells were obtained from Peking Union Medical College (Beijing, China). Both cell lines were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 100 U/ml penicillin and 100 U/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) and supplemented with 10% fetal bovine serum (Biological Industries). All cell lines were incubated at 37˚C in a 5% CO_2 atmosphere.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from EC cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using random primers and M-MLV reverse transcriptase (Promega Corporation) according to the manufacturer's instructions. qPCR was performed using SYBR Premix Ex Taq kit (Takara Bio, Inc.) according to the manufacturer's instructions. The reaction conditions: Pre-denaturation at 95˚C for 2 min; followed by 40 cycles at 95˚C for 15s and 60˚C for 1 min; and melting curve analysis at 95˚C for 1 min, 55˚C for 30 sec and 95˚C for 30 sec. Data were analyzed using the $2^{-\Delta\Delta cq}$ method (32). The primers used were as reported in the literature (28): ISG15 forward, 5'-GCGCAGATCACCCAGAAGAT-3' and reverse, 5'-GTTCGTCGCATTTGTCCACC-3'; and GAPDH forward, 5'-CAAATTCCATGGCACCGTCA-3' and reverse, 5'-GAC TCCACGACGTACTCAGC-3'.

Western blotting. Total protein was extracted from EC cells using RIPA lysis buffer and a protease inhibitor cocktail (Applygen Technologies, Inc.). Protein concentrations were determined using a BCA Kit (Thermo Fisher Scientific, Inc.). A total of 50 μg protein/lane was electrophoresed via 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. Blocking was performed using 5% skimmed milk for 1 h at room temperature. The following primary antibodies were used: Anti-ISG15 (catalog no. ab285367; Abcam), anti-tubulin (catalog no. 5335; Cell Signaling Technology, Inc.), anti-β-actin (catalog no. 3700; Cell Signaling Technology, Inc.) and anti-phosphorylated (p)-retinoblastoma-associated protein (RB1; Ser807/811) (catalog no. 8516; Cell Signaling Technology, Inc.); the antibodies and TBST (20% Tween) were diluted at a ratio of 1:1,000 and incubated overnight at 4˚C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (catalog no. 9003-99-0) were purchased from Applygen Technologies, Inc., diluted in 5% skimmed milk and incubated at room temperature for 1 h. After incubation

with chemiluminescence solution (catalog no. WBKLS0050; Merck KGaA), the signals of the protein bands were acquired using an ImageQuant 800 system (Cytiva).

Small interfering (si)RNA transfection assays. siRNA sequences against human ISG15 mRNA were purchased from Shanghai GenePharma Co., Ltd., and the detailed sequences were downloaded from the published literature (33): si-negative control (NC), 5'-AATTCTCCGAACGTGTCA CGT-3'; siRNA-1, 5'-TCCTGGTGAGGAATAACAA-3'; and siRNA-2, 5'-GGTGGACAAATGCGACGAA-3'. The transfection assay was performed using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 2 μg siRNA was used to transfect the HEC-1A and Ishikawa cells when the cell density reached 80%. At 48 h after transfection, the cells were harvested for RT-qPCR assays and further functional experiments.

Cell proliferation assay. The proliferation rate of cells was determined using the xCELLigence Real-Time Cell Analyzer (RTCA)-MP system (Agilent Technologies, Inc.) according to the manufacturer's instructions. The cell culture well was pre-balanced by adding 50 μl medium; HEC-1A and Ishikawa cells were inoculated into the cell plate, and 3,000 cells in 100 μl medium were then added into each well, and the cell index was measured every h.

Colony formation assay. HEC-1A and Ishikawa cells transfected with ISG15-specific siRNA or control siRNA (si-NC) were inoculated into six-well plates at a density of 1,500 cells per well and cultured for 2 weeks until visible clones appeared. After removing the supernatant, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.1% crystal violet at room temperature. After washing with PBS, images were collected and the results were recorded. Finally, the number of colonies (>50 cells) was counted using Adobe Photoshop 2021 (Adobe Systems, Inc.) Data were normalized to each control group and to 100%.

Cell cycle assay. HEC-1A cells and Ishikawa cells in logarithmic growth phase were resuscitated with complete culture medium and made into suspension with a density of $5x10^4$ per ml. The suspension was inoculated into a petri dish with a diameter of 6 cm according to 3 ml per plate and cultured for 24 h. The cells were divided into three groups: i) si-NC; ii) si-1; and iii) si-2. Three parallel dishes were set up in each group. The cells were collected after 48 h of culture, fixed overnight at 4˚C with 70% ethanol, then washed twice with pre-cooled PBS (pH, 7.4), re-suspended with RNaseA reagent (Beijing Solarbio Science & Technology Co., Ltd.) (100 μl), and incubated in a water bath at 37˚C for 30 min. PI staining solution [500 μl; Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] was added, mixed, incubated for 30 min at 4˚C in the dark, and detected by flow cytometry (FACSCalibur; BD Biosciences), using FlowJoV10 software (BD Biosciences) for data analysis.

Gene Set Enrichment Analysis (GSEA). GSEA was used to determine the effects of ISG15 genes on endometrial cancer-related pathways, with phenotypes marked as positive and negative for ISG15 expression. The number of random combinations of gene sets per analysis was set at 1,000, $P<0.05$ was used to indicate statistical significance and gene sets with a false-discovery rate of <0.25 were considered enriched.

Statistical analysis. All the experiments were repeated three times. All data were analyzed with GraphPad Prism 6 for Windows (GraphPad Software, Inc.) and SPSS for Windows, version 16.0 (SPSS, Inc.). Data are shown as the mean ± SD or mean ± SEM. One-way ANOVA and Bonferroni's correction were performed for multiple comparisons between groups, and Pearson's correlation coefficient was used to determine the correlations between methylation scores and ISG15 mRNA levels, and various cell markers. Spearmen's correlation analysis was also applied. Survival analysis was performed using the Kaplan-Meier method to determine whether the ISG15 gene was associated with survival in patients with endometrial cancer, and P-values were calculated by the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ISG15 is highly expressed and may be a marker of EC tumor progression. The mRNA expression data of ISG15 in normal and tumor tissues for different cancers were obtained from the GEPIA database. As shown in Fig. 1A, ISG15 levels were significantly elevated in cancers, including EC, esophageal squamous cell carcinoma (ESCC), gastric cancer, liver cancer and breast cancer. When all cancer types were ranked according to the mRNA levels of ISG15, most gynecological malignancies, including uterine corpus EC, cervical squamous cell carcinoma and endocervical adenocarcinoma, ovarian cancer and uterine carcinosarcoma, showed elevated ISG15 levels. For EC, the tumor tissues had >10-fold higher ISG15 expression than the corresponding normal tissues (Fig. 1B).

Next, the association between high ISG15 expression and ES subtypes was investigated. ISG15 expression in EC was not significantly associated with age or tumor recurrence, but did differ significantly among molecular subtypes (Fig. S1A). Serous endometrial adenocarcinoma cancer subtypes had the highest level of ISG15 expression, followed by the mixed-type, while adenocarcinoma-type endometrial cancer tissues showed the lowest ISG15 mRNA level (Fig. 1C). Different pathological subtypes of EC have different risk factors and degrees of malignancy (34). As the expression of ISG15 was significantly associated with pathological subtypes, the association between ISG15 expression and EC prognosis was further investigated. It was found that patients with EC with high ISG15 mRNA expression showed significantly shorter overall survival (OS; Fig. 1D).

Next, the protein levels of ISG15 in ECs were evaluated. First, analysis of the proteomic data (Uniprot; http: //www.uniprot.org/) in 100 EC samples and 31 normal samples indicated that the protein levels of ISG15 were also significantly elevated in EC samples (Fig. 1E). Furthermore, IHC of a tissue array of EC and normal tissues showed that 55.9% of EC tissues (19 of 34) expressed notable levels of ISG protein,

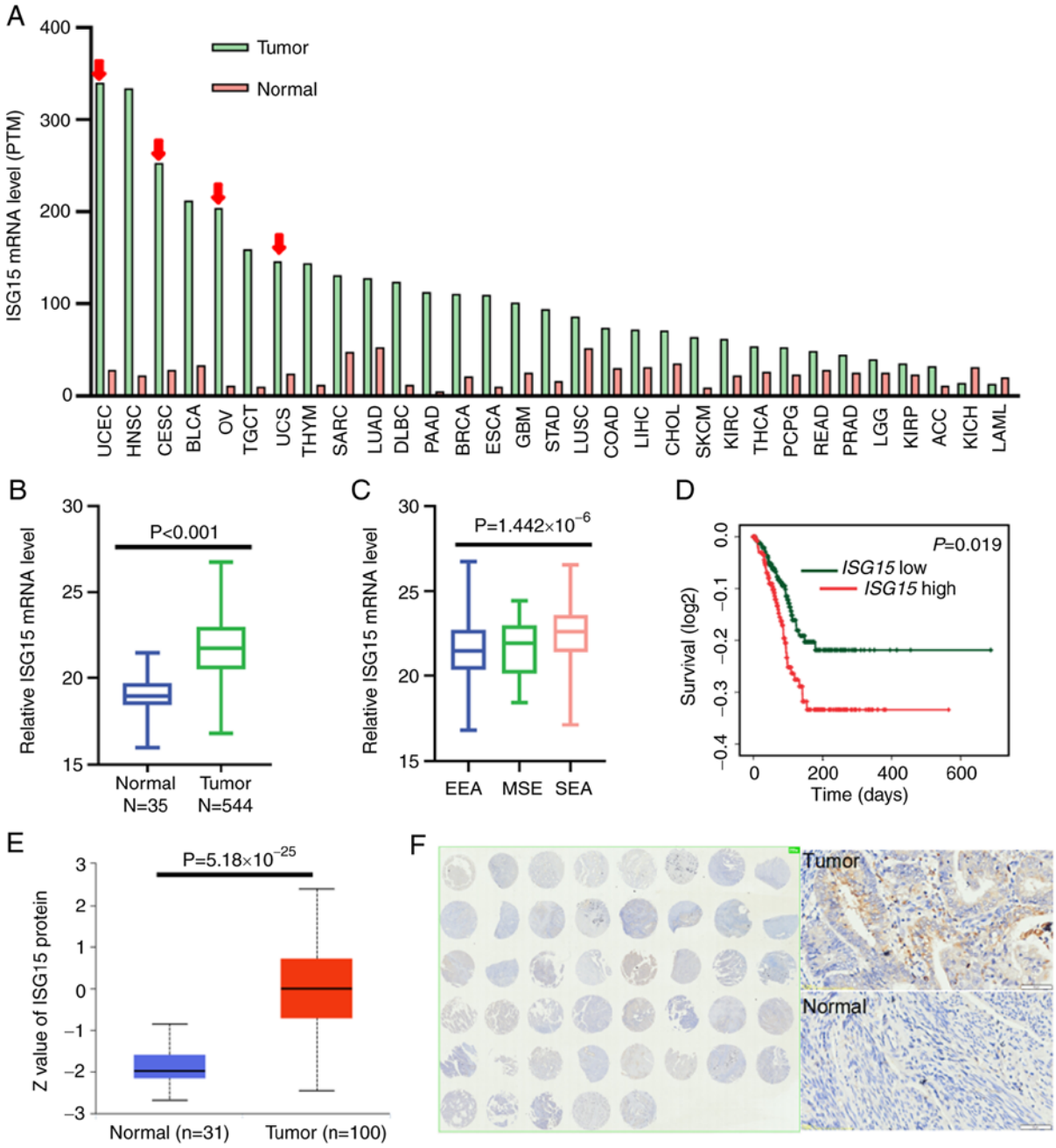

Figure 1. ISG15 is a marker of tumor progression and prognosis in EC. (A) The RNA expression of ISG15 was most significantly increased in gynecologic tumors, with expression levels decreasing from left to right. Gynecological tumors are indicated by the red arrow. (B) mRNA expression levels of ISG15 in tumor vs. normal tissue in cases of EC in TCGA database. Green and blue represent tumor and normal tissue, respectively. (C) mRNA expression levels of ISG15 in different subtypes of EC in cases of EC in TCGA database. (D) Overall survival of patients in the ISG15 high and low expression groups in TCGA EC samples. The mRNA (FPKM) and corresponding clinical information data were downloaded from The Human Protein Atlas and analyzed using SPSS software. Kaplan-Meier survival curve was constructed, which was analyzed using a log-rank test. (E) Protein expression levels of ISG15 in EC based on quantified ISG15 protein data from the UALCAN public database. (F) Representative immunohistochemistry results of ISG15 in EC, showing the results from an EC tissue assay. ISG15, interferon-stimulated gene 15; EC, endometrial carcinoma; TCGA, The Cancer Genome Atlas; FPKM, Fragments Per Kilobase of transcript per Million; UCEC, uterine corpus endometrial carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; OV, ovarian cancer; UCS, uterine carcinosarcoma; EEA, endometrioid endometrial adenocarcinoma; MSE, mixed serous and endometrioid; SEA, serous endometrial adenocarcinoma.

while ISG15 expression could not be detected in all 11 normal endometrial tissues (Fig. 1F).

Taken together, the results of the mRNA and protein expression analyses showed that ISG15 upregulation was most significant in EC, representing a potential tumor marker of EC. Moreover, ISG15 may be a marker of pathological progression of EC with a significant prognostic value.

Abnormal DNA methylation leads to ISG15 upregulation in EC. Next, the molecular mechanisms leading to the upregulation of ISG15 were explored. Surprisingly, increased ISG15 gene copy number was detected in <10% of the samples and there was no significant association between the increased ISG15 copy number and mRNA levels (Fig. 2A). Therefore, ISG15 upregulation in EC was not caused by gene amplification.

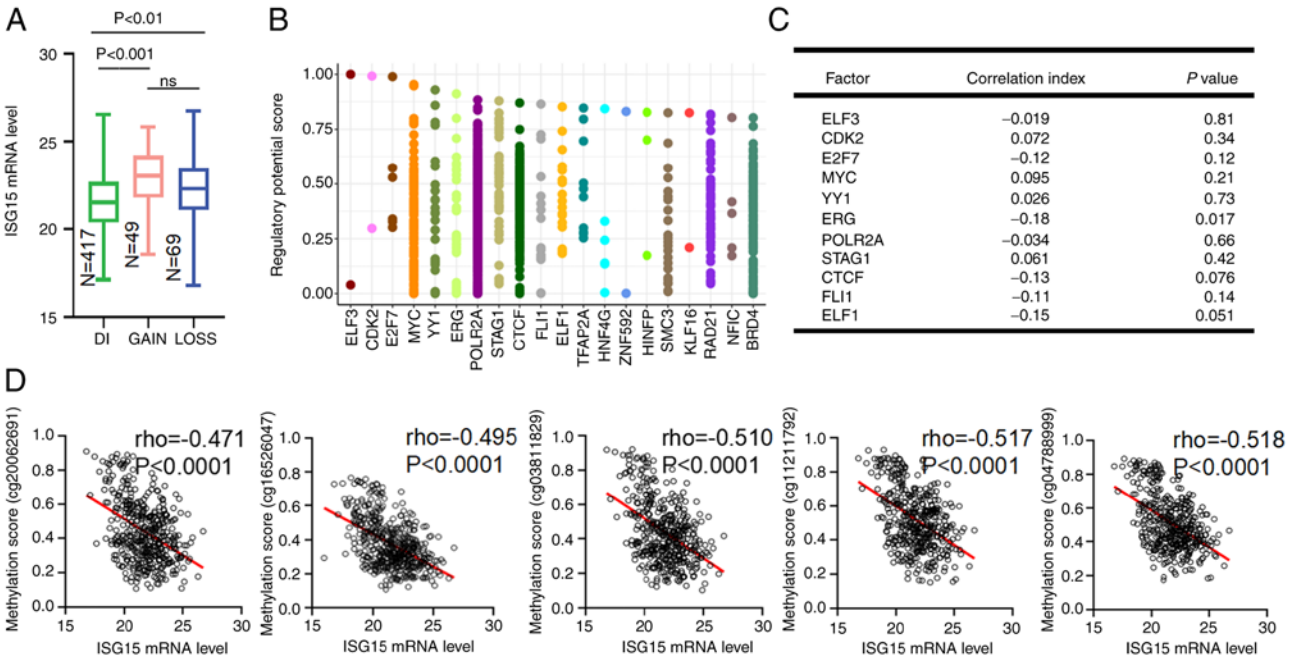

Figure 2. ISG15 upregulation is caused by abnormal DNA methylation. (A) ISG15 expression in patients with endometrial carcinoma with different DNA copy numbers. ISG15 expression and copy number data were downloaded from MEXPRESS. (B) Results of ISG15 transcription factor analysis based on ChIP-seq results. The reliability of each transcription factor binding to the ISG15 promoter was gradually decreased from left to right. The analysis of ChIP-seq data was performed using the Cistrome public database. (C) Correlation analysis between the transcription factors ranking in the top 10 in the feasibility of binding to ISG15 and ISG15 expression. The correlation analysis was performed using the GEPIA public database. (D) Correlations between ISG15 mRNA expression and five CpG methylation sites. The correlation coefficient is also shown. All P-values and R-values were calculated with Spearman's correlation analysis. ISG15, interferon-stimulated gene 15; ChIP-seq, chromatin immunoprecipitation followed by sequencing; GEPIA, Gene Expression Profiling Interactive Analysis.

Then, it was considered whether dysregulation of ISG15-specific transcription factors was involved in ISG15 upregulation. The Cistrome database was used, which allows the analysis of *in vivo* genome-wide location of transcription factor binding sites to screen for transcription factors that may bind to the ISG15 promoter. It was found that a series of well-known tumor-associated transcription factors could bind to this promoter in chromatin immunoprecipitation experiments, including MYC proto-oncogene protein (MYC), E74-like factor 3 and cyclin-dependent kinase 2 (CDK2) (Fig. 2B). However, none of the top 10 transcription factors with the highest binding score to the ISG15 promoter were significantly correlated with ISG15 expression (Fig. 2C). Abnormal DNA methylation plays a regulatory role in the expression of oncogenes in human cancers (35). Notably, the methylation of the CpG sites located in the promoter region of ISG15 also showed no correlation with ISG15 mRNA expression in the present study (Fig. S1B). However, the methylation states of five CpG sites located in the gene body of ISG15 were significantly negatively correlated with ISG15 mRNA levels (R=-0.471 for Chr11014012; R=-0.510 for Chr11014069; R=-0.517 for Chr11014254; R=-0.518 for Chr11014471; and R=-0.495 for Chr11014514; Fig. 2D). Therefore, it was concluded that the upregulation of ISG15 in EC was caused by the dysregulation of DNA methylation in the ISG15 gene body.

ISG15 inhibits T cell infiltration and promotes immune escape. ISG15 is an IFN-stimulating gene. IFN can significantly induce the transcriptional expression of this gene in an inflammatory environment (20). Therefore, in the present study it was considered whether the high ISG15 expression in EC was related to the tumor immune microenvironment. Effective infiltration of $CD8^+$ T cells into tumors is the basis for antitumor immunity. Inhibited infiltration of $CD8^+$ T cells is also a fundamental mechanism for tumor immune escape (36). Correlation analysis showed that the ISG15 expression level was significantly negatively correlated with the number of $CD8^+$ T cells in EC samples; in other words, patients with EC with high ISG15 levels tended to have fewer $CD8^+$ T cells (Fig. 3A), suggesting that it is easier to form an immune microenvironment for 'cold' tumors (without infiltrating lymphocytes). Among T cells that infiltrate the tumor, tumor cells also express T cell inhibitory factors to send an inhibition signal to reduce the killing activity of the T cells (37). In the current study, analysis of the effects of ISG15 on the expression of T cell inhibitory factors showed that ISG15 was highly positively correlated with the expression of most T cell inhibitory molecules, such as programmed death-ligand 1 (PD-L1), indoleamine 2'3-dioxygenase 1 (IDO1) and lymphocyte-activation gene 3 (Fig. 3B). Therefore, the tumor-killing activity of $CD8^+$ T cells was inhibited in ECs with high ISG15 expression.

ISG15 promotes EC cell proliferation and colony formation. Next, the possible function of ISG15 in EC cells was investigated. First, the successful ISG15 knockdown in the two cell lines using ISG15-specific siRNAs was confirmed (Fig. 4A and B). Cell proliferation was measured using the RTCA-MP system. According to the growth curve drawn by the dynamic cell index, the inhibition of ISG15 markedly slowed EC cell proliferation (Fig. 4C), suggesting that ISG15 had a growth-promoting function in EC. The anti-anoikis capacity is vital for tumor formation (38). Therefore, the function of

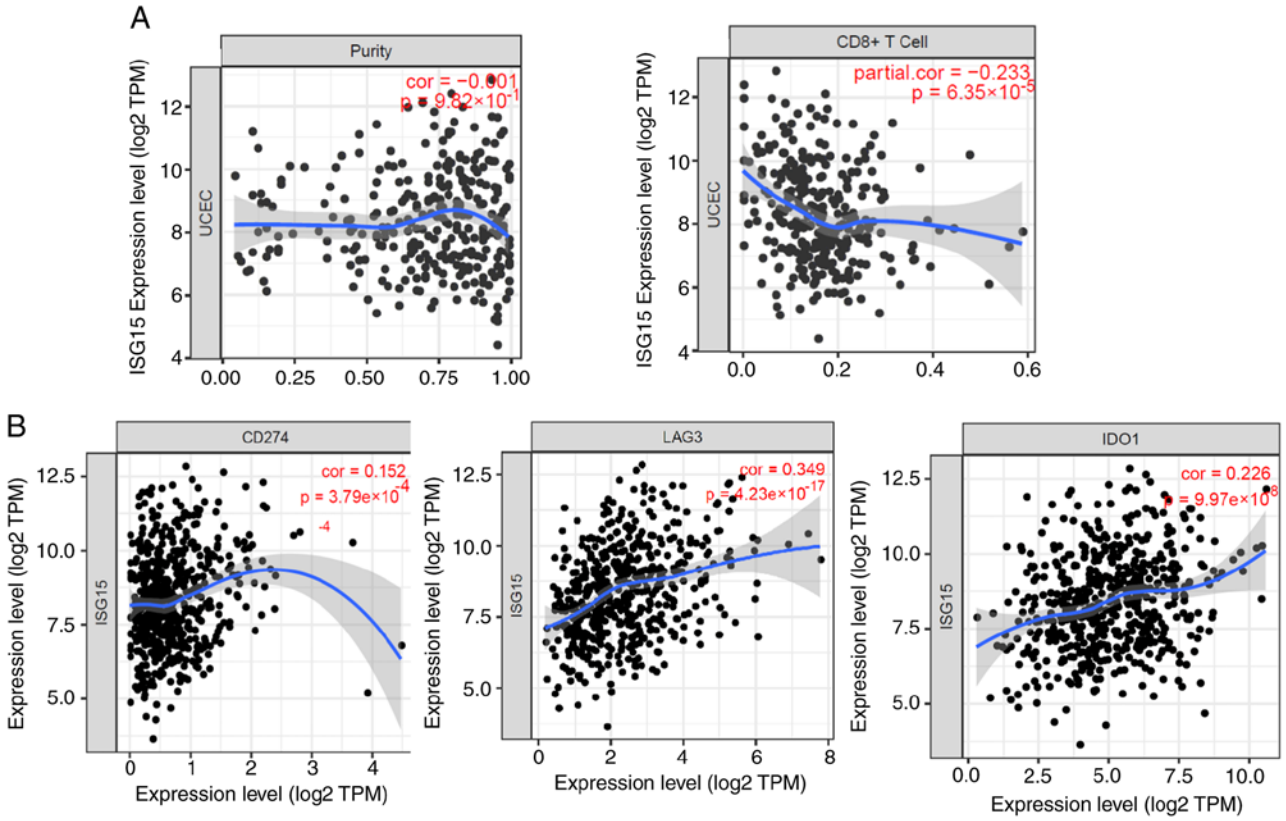

Figure 3. ISG15 negatively regulates tumor immunity. (A) Negative correlations between ISG15 expression and the number of infiltrating CD8+ T cells in endometrial carcinoma. (B) Positive correlations between the expression levels of CD274, LAG3 and IDO1, interferon-stimulated gene 15; IDO1, indoleamine 2'3-dioxygenase 1; ATG3, autophagy-related protein 3; PD-L1, programmed death-ligand 1; LAG3, lymphocyte-activation gene 3; UCEC, uterine corpus endometrial cancer.

ISG15 in cell colony formation was examined. Transfection with ISG15 siRNA resulted in a notable decrease in colony number (Fig. 4D). Taken together, the direct association between ISG15 expression and cell proliferation or colony formation ability indicated the important role of ISG15 in EC tumor cell growth.

ISG15 contributes to EC development via the MYC pathway and cell cycle regulation. The molecular mechanism of ISG15 in promoting EC progression was then explored using Gene Set Enrichment Analysis (GSEA). A total of 14 and nine pathways were positively and negatively correlated with ISG15 expression, respectively (Tables SII and SIII). In addition to the well-studied ISG15-associated pathway in immune responses, such as IFN-α response and IFN-γ response signaling pathways (39) (Fig. 5A and B), the 'hallmark_MYC_targets_V2' pathway was also evaluated as MYC target activation is involved in numerous processes necessary for cancer development, especially cell proliferation (40). It was found that MYC expression was significantly associated with ISG15 expression (Fig. 5C). Thus, ISG15 may function via the MYC pathway. Additionally, the activation of MYC targets is generally considered a marker of cell cycle activation (40). Therefore, the role of ISG15 in the cell cycle was investigated. The results showed that ISG15 knockdown increased the number of cells in the G1/G0 phase of both EC cell lines (Fig. 5D and E), which may be responsible for the inhibition of EC cell proliferation with a low expression of ISG15. To further confirm the function of ISG15 in the cell cycle G1/S transition, the expression of p-RB1, a standard protein marker representing the activation of the cell cycle G1/S transition (41), was examined. As shown in Fig. 5F, ISG15 knockdown reduced the expression of p-RB1 and proliferating cell nuclear antigen (PCNA) in both EC cell lines. According to TCGA tumor mRNA expression database, ISG15 was significantly positively correlated with G1/S-specific cyclin-D3 (CCND3), G1/S-specific cyclin-E1 (CCNE1), CDK4 and transcription factor E2F1 (E2F1) (R=0.37 for CCND3; R=0.35, P<0.001 for CCNE1; R=0.20 for CDK4; and R=0.31 for E2F1; Fig. 5G). Taken together, these results suggested that ISG15 promoted the G1/S cell cycle transition in EC cells via the MYC pathway.

Discussion

Despite advances in diagnostic and clinical management, the prognosis of EC remains poor (35,42). The molecular mechanisms underlying EC progression remain unclear. Thus, there is a need to further explore the molecular mechanisms underlying the occurrence and development of EC and identify new and meaningful prognostic markers. ISG15, a UBL protein involved in protein modification, reportedly plays an essential role in tumor development. Some investigations have revealed a strong association between aberrant ISG15 expression and the incidence of some human malignancies, including esophageal squamous cancer and liver cancer (43,44). However, the

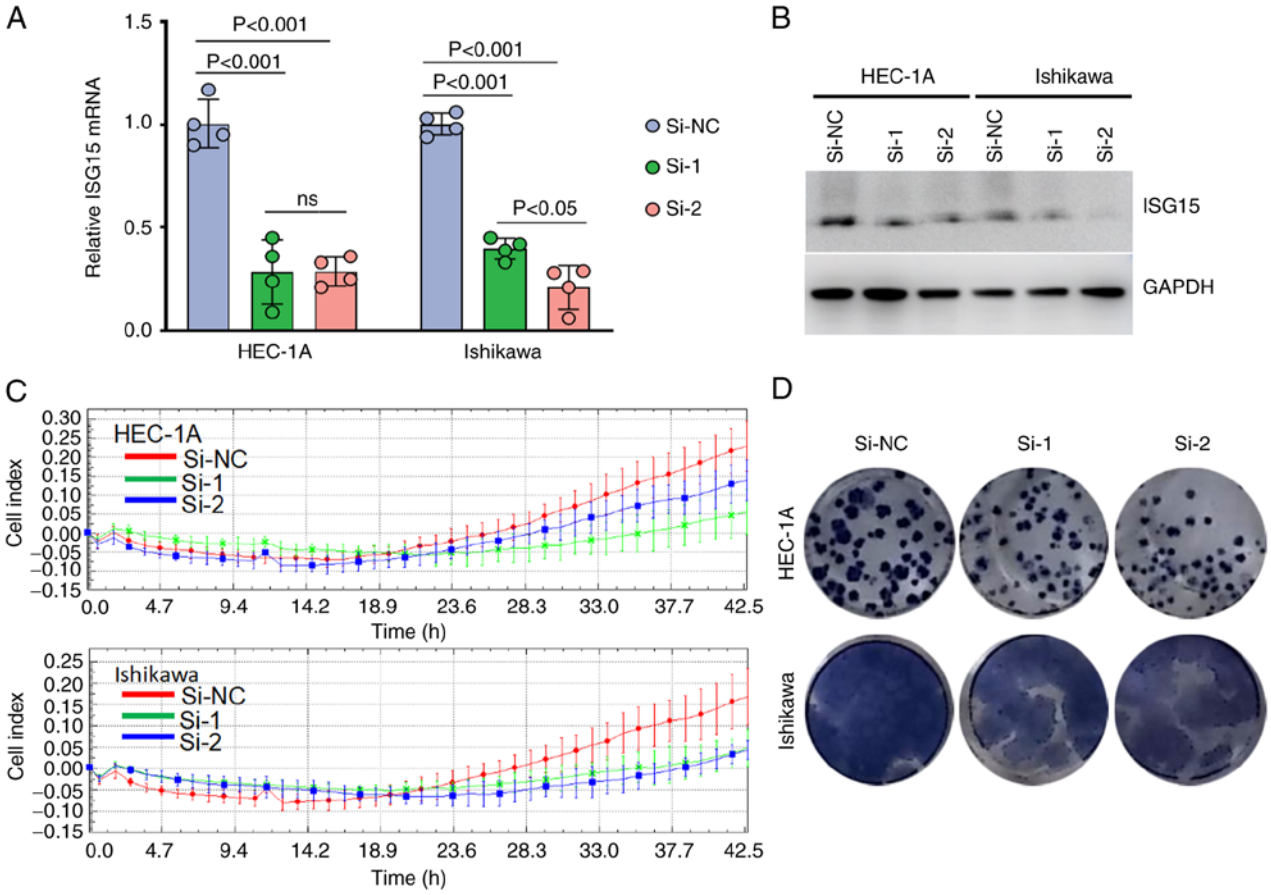

Figure 4. ISG15 regulates the proliferation of EC cell lines. (A) ISG15 mRNA levels in EC cell lines transfected with ISG15-specific siRNA (si-1 and si-2) or control siRNA (si-NC). (B) Immunoblotting of ISG15 in EC cell lines transfected with ISG15-specific or control siRNA. (C) Real-time cell analysis-multiple plate proliferation assay of EC cells transfected with ISG15-specific or control siRNA. (D) Clone formation of EC cell lines transfected with ISG15-specific or control siRNA. All P-values were calculated with one-way ANOVA. ISG15, interferon-stimulated gene 15; EC, endometrial carcinoma; siRNA, small interfering RNA; NC, negative control.

relative expression status and function of ISG15 in ECs remain to be elucidated.

In the present study, the upregulation of ISG15 in EC was demonstrated by re-analyzing the public transcriptome sequencing, proteomics and IHC data. In addition, the Kaplan-Meier survival curves showed the association of high ISG15 levels with an unfavorable prognosis. These results suggested that ISG15 may play an important role in the development of EC. It is worth noting that the expression of ISG15 in tumors was specific to gynecologic tumors. Among the five types of tumors with the highest ISG15 expression levels, three were gynecologic tumors (EC, cervical cancer and ovarian cancer), with EC ranking first. The occurrence and progression of gynecologic tumors are closely related to abnormal hormone levels (45), suggesting a regulatory relationship between ISG15 and hormones, which requires clarification in future studies.

The results of the clinical correlation analysis in the current study showed that elevated ISG15 expression was highly correlated with the pathological stage (Fig. S1C), indicating that ISG15 may be a promising marker of EC progression. Furthermore, it was found that ISG15 knockdown significantly inhibited the malignant phenotypes of EC cells, including proliferation and colony formation, suggesting the central role of ISG15 in the malignant progression of EC cells.

Furthermore, as ISG15 is an IFN-induced gene, the relationship between ISG15 and immunity status in EC was analyzed. It was found that ISG15 was negatively correlated with $CD8^+$ T cell infiltration and positively correlated with CD274, ATG3 and IDO1. PD-L1, also called B7-H1 or CD274, is a ligand of PD-1. This axis affects immune checkpoints to mediate T cell depletion, characterized by a loss of cytokines, impaired proliferation, lack of cytotoxic activity, and, ultimately, inhibition of an effective immune response (46). In cancer, PD-L1 upregulates the immune defense of similarly hijacked hosts to promote T cell depletion, resulting in immune resistance (47-49). ATG3 is a key gene involved in autophagy and its homologous genes are common in eukaryotes. Lawson *et al* (50) screened the whole-genome Clustered regularly interspaced short palindromic repeats of Renca cells and restored the established suppressors. Cytotoxic T lymphocyte (CTL) escape mutations in numerous types of cancers were identified in this screen, including genes annotated in the autophagy pathway (ATG3, ATG5, ATG7, ATG10, ATG12 and ATG14), confirming the importance of IFN-γ response to the innate CTL escape phenotype (50). The study showed that disturbances in autophagy and peroxisome pathways are some of the most sensitive mutations in wild-type Renca cells when IFN-γ (such as interferon-induced transmembrane protein 2) is used alone. These results highlight the profound

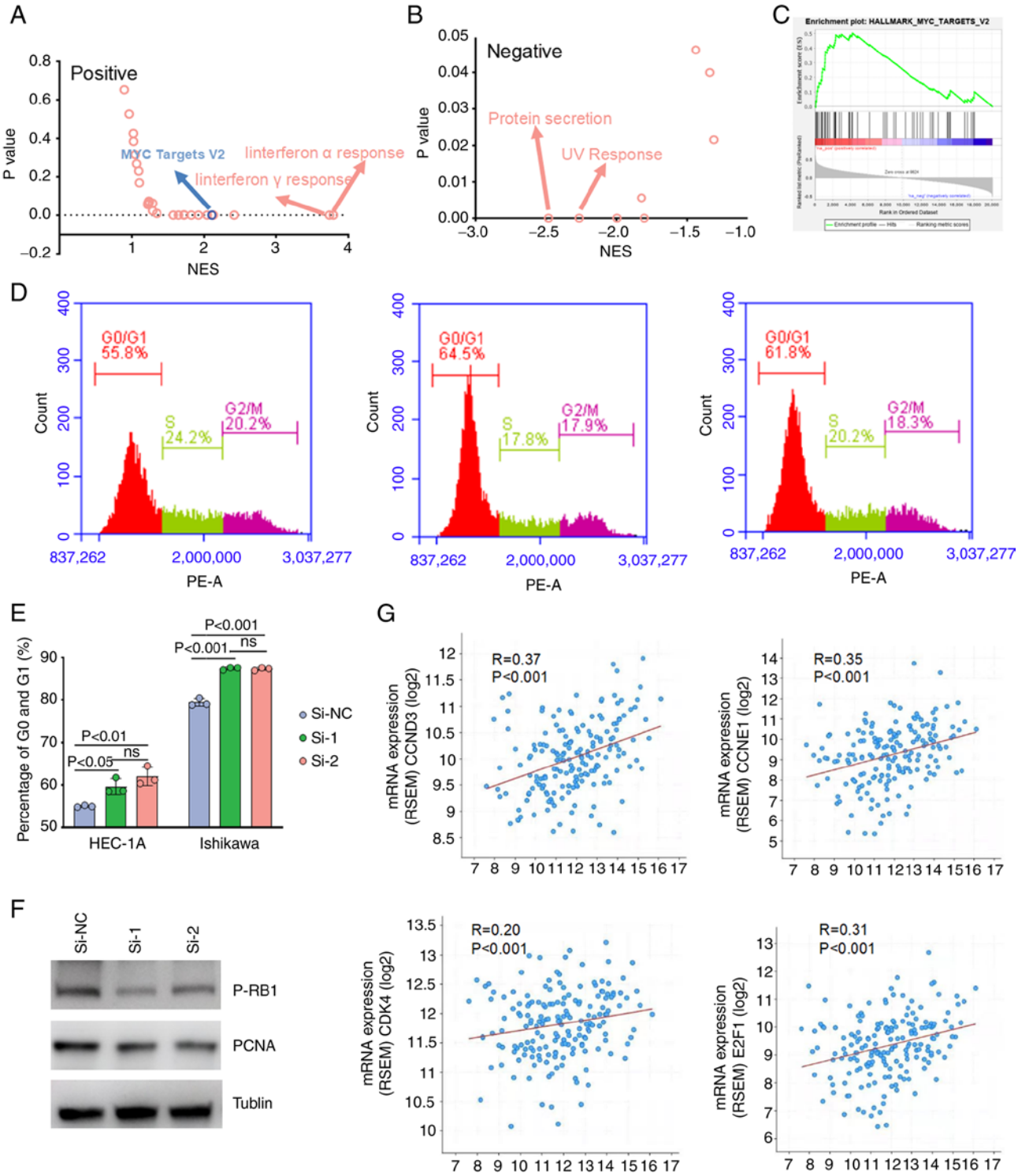

Figure 5. Enriched pathways associated with ISG15. The scatter diagrams depict the hallmark pathways (A) positively and (B) negatively associated with high ISG15 levels. The x- and y-axes show the NES and P-values, respectively. (C) Gene Set Enrichment Analysis results for ISG15 expression and 'hallmark_MYC_targets_V2' pathway. (D) Representative cell cycle profile images of HEC-1A cells transfected with specific or control siRNA. (E) Quantification of G0 and G1 cell percentage in endometrial carcinoma cells transfected with specific or control siRNA. (F) Detection of p-RB1 and PCNA expression in endometrial cancer cells transfected with without siRNA by western blotting. (G) Scatter diagrams showing the positive correlation between cell markers responsible for the G1/S transition. All P-values and R-values were calculated with one-way ANOVA and Pearson's correlation analysis. ISG15, interferon-stimulated gene 15; NES, normalized enrichment scores; MYC, MYC proto-oncogene protein; siRNA, small interfering RNA; NC, negative control; CCND3, G1/S-specific cyclin-D3; CCNE1, G1/S-specific cyclin-E1; E2F1, transcription factor E2F1.

effects of the autophagy pathway in regulating the inherent CTL escape of cancer cells (50). The inhibition of autophagy also affects some aspects of the immune system, such as the memory formation of virus-specific $CD8^+$ T cells and the activation of $CD4^+$ T cells by DCs, which may inhibit the death of immunogenic cells induced by chemotherapy (51). IDO1 is a cytoplasmic monomer enzyme-containing heme, the most crucial inducer of which is the cytokine IFN-γ. IDO1

is considered an effector molecule that can mediate a survival strategy based on tryptophan deprivation and catalyze the initial rate-limiting step of the degradation of the essential amino acid tryptophan in the canine pathway, through which IDO1 plays an essential role in maintaining maternal T cell tolerance (52). Thus, IDO1 is now considered an immunomodulator in autoimmune diseases, chronic inflammation and tumor immunity (53). Taken together with the present results, it can be speculated that high ISG15 expression negatively regulates antitumor immunity and is positively associated with tumor immune escape.

ISG15 functions not only as a modifier of target proteins, but also as a free protein that promotes cancer progression (54,55). ISG15 can conjugate with Ki-Ras to reverse the malignant phenotypes of breast cancer (56). Falvey *et al* (57) reported that the depletion of ISG15 expression promoted autophagy. Thus, ISG15 is also a novel inhibitor of autophagy, potentially influencing 5-Fluorouracil-mediated chemosensitivity in esophageal cancer cells (43). High ISG15 mRNA expression has been observed in ESCC tissues and may serve as a novel prognostic biomarker for ESCC among alcohol drinkers (43). However, the specific mechanism of ISG15 in EC has not yet been fully elucidated. In the present study, the potential molecular mechanism by which ISG15 promotes tumorigenesis was explored. The combination of data from bioinformatics analysis, flow cytometry and immunoblotting analyses indicated that ISG15 regulated the G1/S transition and cell proliferation via the MYC pathway. However, the function of ISG15 as a free protein or protein-modifying factor in EC requires further investigation.

DNA methylation is indicative of gene expression, with gene body methylation being a more useful indicator than promoter methylation (58). The results of the current study showed that ISG15 upregulation in EC was caused by a dysregulation of DNA methylation in the ISG15 gene body, suggesting that ISG15 deregulation in EC is related to DNA methylation and that regulation of methylation may be a strategy to decrease ISG15 expression.

Our research work has certain reference significance for understanding the pathogenesis of EC, including cell proliferation immune escape. The PTM of proteins has gradually become a research hotspot in the development of tumor-targeted therapeutic drugs (59). Considering the significant clinical value of ISG15 in EC, we speculate that ISG15 may also be a promising therapeutic target for EC in the future. However, the current study has certain limitations. For example, the investigation of the methylation modification of the ISG15 promoter region was limited at the phenotypic level in its infancy, and the experiments investigating the immune escape of ISG15 lacked further cytology and animal experiments. Thus, we will perform animal experiments to further verify the immunotherapy value of ISG15, and explore the detailed mechanism of ISG15 in upstream and downstream pathways in different types of EC cells.

In conclusion, the results of this study demonstrated that dysregulation of the methylation of ISG15 led to its high expression and was related to poor clinical outcomes and pathological stage of EC. The knockdown of ISG15 expression attenuated the malignant phenotypes of EC cell lines. Moreover, it was also found that the MYC pathway was part of the potential mechanism through which ISG15 promoted EC tumorigenesis.

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Availability of data and materials

This datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and XZ conceived and designed the experiments. XZ and JW completed the experiments and explained the experimental data. YW and MZ collected specimens for the experiments and participated in the drafting of the manuscript. WZ and HZ analyzed the database information and substantially revised the content of the manuscript. LZ and XZ integrated all experimental data for data analysis and statistical analysis and calibrated the publication of the final version. LZ and XZ confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). All patients who contributed to the research provided their written consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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