



CD73 promotes cervical cancer growth via EGFR/AKT1 pathway

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Contributions: (I) Conception and design: K Dong, HZ Zhang; (II) Administrative support: F Lin; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: X Wang; (V) Data analysis and interpretation: ZW Gao; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Background: Cervical cancer ranks third in cancer incidence worldwide and is the most frequent gynecological cancer in developing countries. To explore the molecular mechanism of cervical cancer and to find effective treatment have become the focus of medical workers. CD73 has been implicated in the progression of many cancers. However, the study of CD73 in cervical cancer has not been reported. The aim of this study was to identify the effect and mechanism of CD73 overexpression on cervical cancer growth *in vitro* and *in vivo*.

Methods: Cervical cancer cell models with CD73 overexpression were construction by using lentiviruses infection in HeLa and SiHa cells. Cell's proliferation was investigated by using xCELLigence real-time cell analysis (RTCA) system. Murine xenograft models were used to evaluate the effect of CD73 overexpression on tumor growth *in vivo*. Small interfering RNA (siRNA) transfection were used to suppress expression levels of EGFR and AKT1. Cell cycle and apoptosis were evaluated by flow cytometry (FCM).

Results: CD73 overexpression significantly promoted cervical cancer cells proliferation *in vitro* and tumor growth *in vivo*. The expression levels of EGFR and AKT1 were significantly increased in cell models and transplanted tumor tissues with CD73 overexpression. And moreover, knockdown of EGFR and AKT1 could inhibit proliferation of CD73 overexpressed cell models via inducing cell apoptosis and cell cycles increased in G2/M phase and reduction of G1 phase. Furthermore, the expression levels of CDK2, CDK3 and CDKN1A, which are cell cycle regulated molecules, were significantly increased in CD73 overexpressed cells with EGFR/AKT1 knockdown.

Conclusions: Our data demonstrated that CD73 overexpression promote cervical cancer growth *in vitro* and *in vivo*, via activating EGFR/AKT1 pathway.

Keywords: CD73; cervical cancer; growth; EGFR; AKT1

Submitted Nov 04, 2021. Accepted for publication Mar 10, 2022.

doi: 10.21037/tcr-21-2446

View this article at: <https://dx.doi.org/10.21037/tcr-21-2446>

Introduction

CD73 is a 70 kd, glycosylphosphatidylinositol (GPI) anchored cell surface protein, also known as ecto-5'-nucleotidase (ecto-5'NT, EC 3.1.3.5), which is encoded by NT5E gene (Gene ID: 4907) (1). CD73 is high expressed in various types of tumor cells, which has been found to be correlation with tumor cell's proliferation, adhesion, migration and invasion (2-6). And moreover, multiple

retrospective studies have showed that CD73 overexpression is correlated with clinical stages, metastasis, prognosis of cancer patients (7-13). In preclinical experiments, CD73-blocked have showed the anti-tumor effects (14-16). CD73 has both enzymatic and non-enzymatic functions. As an enzyme, CD73 catalyzes the hydrolysis of AMP into adenosine and phosphate (17). In addition to its enzymatic function, CD73 have been found to be a regulatory

Table 1 Prime sequences used in the study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CD73	GCCTGGGAGCTTACGATTTTG	TAGTGCCCTGGTACTGGTCCG
EGFR	CCAAGGCACGAGTAACAA	ACATAACCAGCCACCTCC
AKT1	ACTGTCATCGAACGCACCTT	TTCTGCAGGACGCGGTTCTC
CDK1	AGCTTTTGAATACCTATCAG	AAAAGTGGTTTCTTAGTTGCT
CDK2	GCCAGAAACAAGTTGACGG	ATGAGGGGAAGAGGAATGCCAGT
CDK3	TAAGTGGACCAGGAAGGGAC	GGGCAGTCTTGGCTGTGAT
CDK4	CGACCAGTTGGGCAAAATC	CCCGACTCCTCCATCTCAG
CDK6	CCAGCAGCGGACAAATAAA	CTGCAAATATGCAGCCAACACT
CDK7	TAGGGATCTGAAACCAAACA	ACCATACATCCTAGCTCCAA
CDKN1A	GGGGACAGCAGAGGAAGAC	CGGCGTTTGGAGTGGTAG
CDKN1B	GGGGCTCCGGCTAACTCTGA	GCAGGTGCGTTCCTATTCC
GAPDH	GGTGGTCTCCTCTGACTTCAACAG	GTTGCTGTAGCCAAATTCGTTGT

molecule which related to tumor cell's proliferation and metastatic properties, while the mechanism is unclear (18). In our previous studies, we found that CD73 overexpression might be correlated with EGFR/AKT1 signal pathway in cervical cancer cells (3). In this present study, we further verified the functions and possible mechanism of EGFR and AKT1 in cervical cancer cells with higher CD73 expression level. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://tcr.amegroupp.com/article/view/10.21037/tcr-21-2446/rc>).

Methods

Cell culture

Human cervical cancer cell lines HeLa (RRID:CVCL_0030) and SiHa (RRID:CVCL_0032) was preserved by clinical Laboratory (Tangdu Hospital, Air Force Military Medical University, China). The cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, Carlsbad, NY, USA) medium plus 10% heat-inactivated fetal bovine serum (FBS, SijiqingBiotec, Hangzhou, China) at 37 °C with 5% CO₂ in a humidified incubator.

CD73 lentivirus packaging and stable cell models construction

CD73 encoding gene sequence was cloned by Polymerase chain reaction (PCR). Then, CD73 lentivirus plasmid were

constructed by ligating cloned fragment to lentivirus vector (pLenti-C-Myc-DDK-IRES-Puro). The CD73 lentivirus plasmid and two helper plasmids (PAX2 and VSVG) were transfected into HEK293T cells, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cell supernatant were collected and concentrated after 72 h. The recombinant lentivirus was stored at -80 °C, named CD73/Lentivirus. HeLa and SiHa cells were infected with CD73/Lentivirus. Stable cell models were screened with 5 µg/mL puromycin.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was carried out using FastStart Essential DNA Green Master kit (Roche) and was used to detect the mRNA expression levels. The primer sequences were list in *Table 1*.

Flow cytometry (FCM)

FCM were used to evaluate the CD73 expression. In brief, Cells were obtained and resuspended in PBS buffer at a concentration of 1×10⁵ cells/mL, and then incubated with fluorescein isothiocyanate (FITC)-labeled anti-CD73 antibody (CD73/FITC) for 30 min at 37 °C. Then cells were washed twice with PBS. Following that, cells were analyzed using FACS calibur flow cytometer (BD Biosciences, San Jose, USA).

Table 2 siRNA sequences used in the study

Name	Sense	Antisense
si-EGFR	GCAGAGGAAUUAUGAUCUUTT	AAGAUCAUAAUCCUCUGCTT
si-AKT1	AGGAAGUCAUCGUGGCCAATT	UUGGCCACGAUGACUCCUTT
NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Small interfering RNA (siRNA) transfection

EGFR- and AKT1-targeted siRNA and irrelevant control siRNA (NC) were synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China). The siRNA transfection was performed using Lipofectamine 2000. siRNA sequences were listed in *Table 2*.

Cell proliferation assay

Cell proliferation was detected by using a xCELLigence system (RTCA Analyzer DP version, ACEA Biosciences). In short, 1×10^4 cells in each well were used for RTCA assay. First, 50 μ L DMEM with 10% FBS were added in each well of E-plate for baseline determination. After baseline determination, 1×10^4 cells in DMEM with 10% FBS were added in E-plate wells, then, E-plate was placed in the RTCA analyzer at 37 °C in a 5% CO₂ humidified incubator. Impedance measurements at every 5 min for 72–96 h. Data of cell proliferation were analyzed via RTCA-soft (ACEA Biosciences).

Cell cycle analysis

Cell cycle was analyzed by ethanol-fixed cells stained with propidium iodide (PI) in buffer containing RNase A. The DNA content was assessed by using FACS caliber flow cytometer (BD Biosciences). Cell cycle analysis was performed 48 h after siRNA transfection.

Cell apoptosis analysis

Cell apoptosis was detected using the annexin V-FITC apoptosis detection kit (KeyGENBioTECH, Nanjing, China). Briefly, cells were collected and washed with PBS twice, stained for 15 min at room temperature with annexin V-FITC and PI. Following that, cell apoptosis was examined using FACS caliber flow cytometer (BD Biosciences). The percentage of apoptotic cells was calculated with CellQuest pro. Cell apoptosis analysis was performed 48 h after siRNA

transfection. Statistical analysis of late apoptotic cells double-stained with annexin V-FITC and PI.

Animal preparation

Twenty 6-week-old female BALB/c nude mice (RRID: IMSR_JCL: JCL: mID-0001) were purchased and fed by animal Center of Air Force Military Medical University for tumor xenogenesis experiment. The mice were raised in groups under controlled conditions, each group was kept in individually ventilated cages at a temperature of 20 to 24 °C, humidity of 50% to 60%, and a 12 h light/dark cycle, with *ad libitum* access to mice chow.

Murine xenograft models

The effect of CD73 overexpression on tumor growth *in vivo* was assessed by subcutaneous injection of 4×10^6 HeLa cells infected with CD73/Lentivirus or control lentivirus into BALB/c nude mouse. Twenty mice were randomly assigned to four groups, five female mice were in each group (age: 6–7 weeks; body weight: 20–25 g). The tumors grew to approximately 40 mm³ to initiate the tests, tumor size was measured every 5 days with a vernier caliper for 25 days. The maximum size the tumors allowed to grow was 2,000 mm³. The mice were sacrificed by neck dislocation, and the mice were confirmed dead without activity. The volume of tumor was calculated according to the formula: $a \times b^2 \times 0.5$ (a , largest diameter; b , perpendicular diameter). All procedures of animal experiments were processed according to animal welfare guidelines approved by Air Force Medical University's Institutional Animal Care and Use Committee.

Statistical analysis

All data were confirmed in three biological replicates. Data were calculated and presented as the mean \pm SD. ANOVA and Tukey test were used to perform comparisons of multiple groups. Comparison between two groups

were conducted by using the Student's *t*-test. $P < 0.05$ was considered to be statistical significant. All analysis was performed with R software (version 3.4.2).

Ethical statement

Experiments were performed under a project license (No. 20200505) granted by Air Force Medical University's Institutional Animal Care and Use Committee, in compliance with Air Force Medical University's guidelines for the care and use of animals.

Results

Overexpression of CD73 promote cervical cancer cells proliferation *in vitro* and *in vivo*

Hela and SiHa cells were infected with CD73/lentivirus or negative control lentivirus, followed by screened with puromycin. The stable cell models with CD73 overexpression were named Hela^{CD73} and SiHa^{CD73}. Compared with control cells (Hela^{NC} and SiHa^{NC}), the expression levels of CD73 were significantly increased in Hela^{CD73} and SiHa^{CD73} cells (Figure 1A-1D). Then the effect of CD73 overexpression on cervical cancer cells proliferation was examined. RTCA results showed that the proliferation rate of Hela^{CD73} and SiHa^{CD73} cells were higher than control cells (Figure 1E,1F). Following, Hela^{CD73} and Hela^{NC} cells were injected subcutaneously into athymic nude mice to construct murine xenograft models. The results showed that tumor growth rate was significantly faster in CD73 overexpressed xenograft models than that in control group (Figure 1G,1H). Taken together, CD73 overexpression could promote cervical cancer cells proliferation *in vitro* and tumor growth *in vivo*.

EGFR and AKT1 play important role in growth of Hela^{CD73} and SiHa^{CD73} cells

Previous study has shown that CD73 overexpression was positive correlation with EGFR and AKT1 expression. Consistently, in this study, we found that the expression levels of EGFR and AKT1 were increased, both in CD73 overexpressed cancer cell models and transplanted tumor tissues (Figure 2A-2C). Furthermore, to verify the function of EGFR and AKT1 expression level on proliferation of CD73 overexpressed cervical cancer cells, the transient transfection of si-EGFR and si-AKT1 was used to suppress

EGFR or AKT1 expression in Hela^{CD73} and SiHa^{CD73} cells (Figure 2D,2E). RTCA experiments showed that knockdown of EGFR and AKT1 could significantly decrease the proliferation rate of Hela^{CD73} and SiHa^{CD73} cells (Figure 2F,2G). The results suggested the important roles of EGFR and AKT1 signal in increased proliferation rate of CD73 overexpressed cervical cancer cells.

Interference of EGFR and AKT1 affects cell cycles and apoptosis of CD73 overexpressing cells

To understand the potential mechanism of EGFR and AKT1 in promoting proliferation of CD73 overexpressed cervical cancer cells. We investigated the effects of EGFR and AKT1 on cell cycle and apoptosis of Hela^{CD73} and SiHa^{CD73} cells. First, the results showed that knockdown of EGFR and AKT1 led to cell cycle reduced in G1 phase and increased in G2/M of Hela^{CD73} and SiHa^{CD73} cells (Figure 3A,3B). And moreover, we investigated the expression levels of cell cycle related molecules. The results showed that EGFR and AKT1 knockdown significantly promote expression level of CDK2, CDK3 and CDKN1A (Figure 3C,3D). Second, FCM results showed that knockdown of EGFR and AKT1 could promote Hela^{CD73} and SiHa^{CD73} cells apoptosis (Figure 4A,4B). Compared with control cells, the apoptosis rate of EGFR and AKT1 knockdown cells increased 2-3 times ($P < 0.05$). Taken together, these results demonstrated that knockdown of EGFR and AKT1 inhibited Hela^{CD73} and SiHa^{CD73} cells proliferation via inducing of cell cycles arrest and cell apoptosis.

Discussion

CD73 is widely expressed on the surface of various tissues and cells in the human body, including lymphocytes, endothelial cells, and epithelial cells. CD73 is involved in multiple biological progress such as immune regulation, cell adhesion, angiogenesis (19,20). CD73 have been found to be a key regulatory molecule in development of cancer, and is overexpression in a variety of cancer cells and patients' biopsies (21). In our studies, we showed that CD73 overexpression promoted cervical cancer cells proliferation *in vitro* and tumor growth *in vivo*.

EGFR/Akt signal play important roles in tumor cell's biological progression, such as proliferation, invasion, apoptosis, etc. Thus, EGFR/Akt signal has been showed to be involved in development of various tumor types,

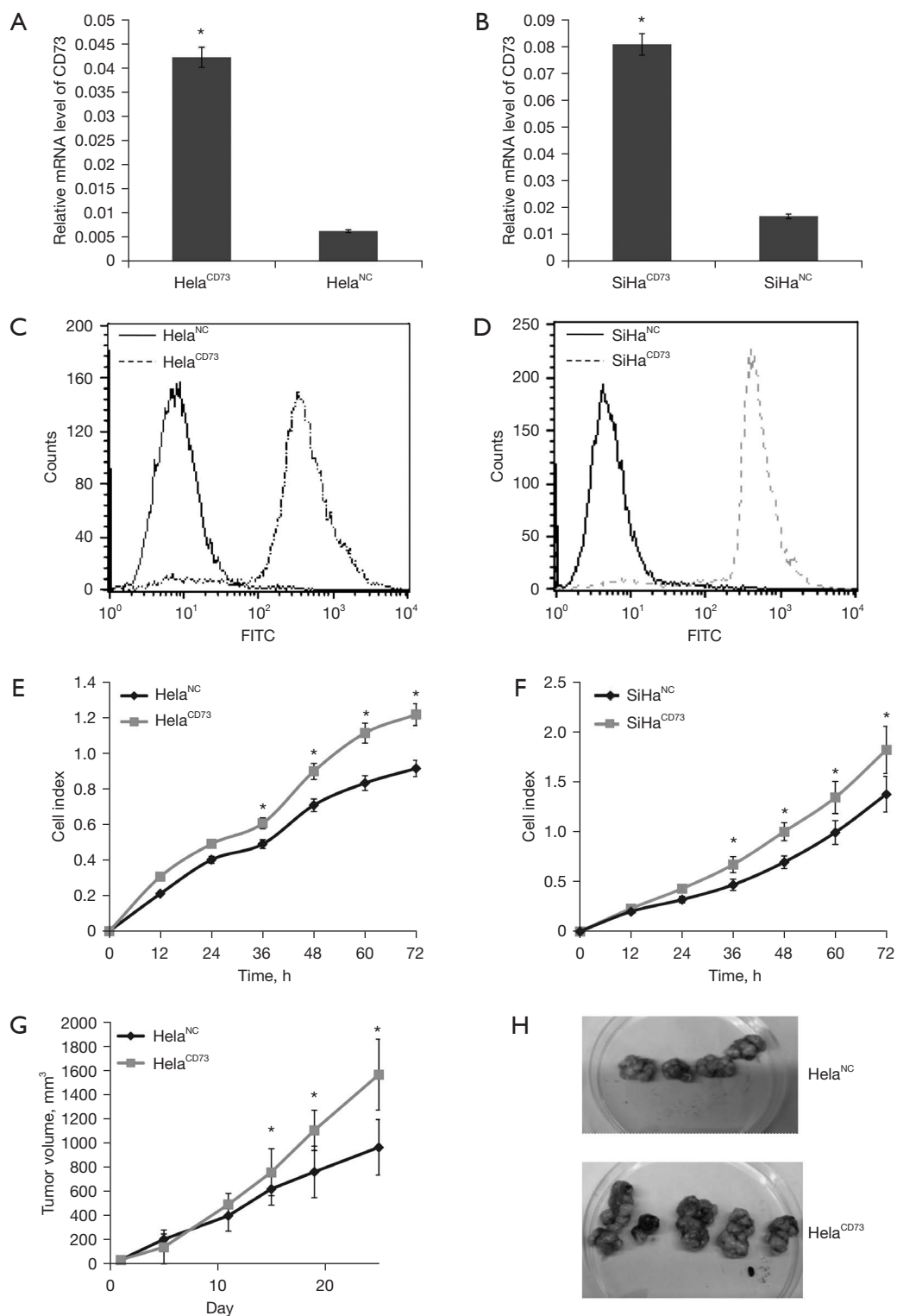


Figure 1 Construction of CD73 overexpressed cell models and the effect of CD73 overexpression on cervical cancer growth. (A-D) qRT-PCR and FCM showed the higher mRNA and protein expression level of CD73 in HeLa^{CD73} and SiHa^{CD73} cells. (E,F) RTCA showed the increased proliferation rate of HeLa^{CD73} and SiHa^{CD73} cells *in vitro*. (G,H) Nude mice models showed that CD73 overexpression promoted tumor growth *in vivo*. *, P<0.05. qRT-PCR, quantitative real-time polymerase chain reaction; FCM, flow cytometry; RTCA, real-time cell analysis.

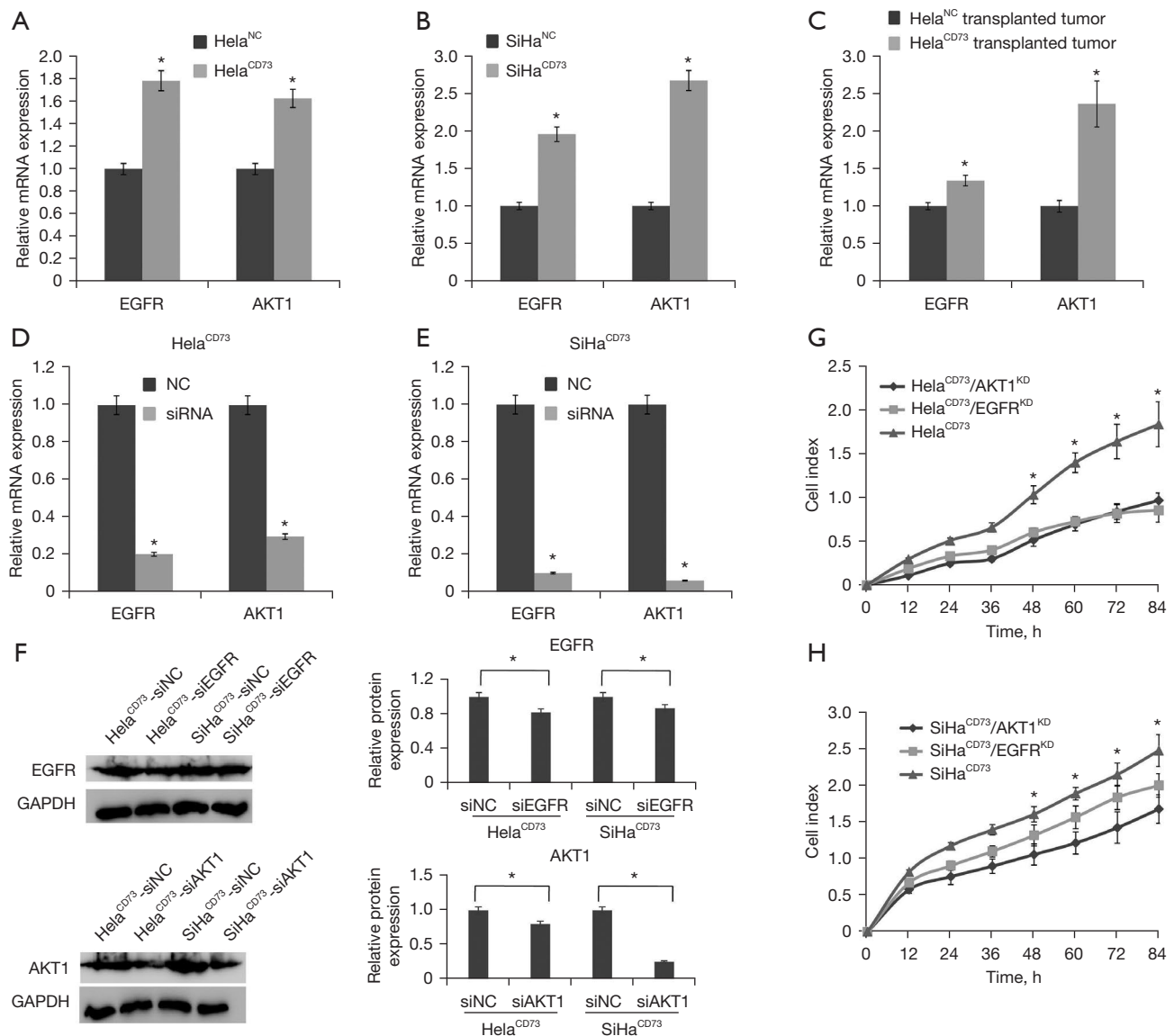


Figure 2 The effects of EGFR and AKT1 expression in HeLa^{CD73} and SiHa^{CD73} cells proliferation. (A,B) Expression levels of EGFR and AKT1 were increased in HeLa^{CD73} and SiHa^{CD73} cells. (C) In compared with HeLa^{CD73} transplanted tumor tissues, the expression levels of EGFR and AKT1 were increased in HeLa^{CD73} transplanted tumor tissues. (D,E) The mRNA expression levels of EGFR and AKT1 were decreased in HeLa^{CD73}/SiHa^{CD73} cells by transfection of si-EGFR and si-AKT1. (F) The protein expression levels of EGFR and AKT1 were decreased in HeLa^{CD73}/SiHa^{CD73} cells by transfection of si-EGFR and si-AKT1. (G,H) Knockdown of EGFR (EGFR^{KD}) and AKT1 (AKT1^{KD}) inhibited HeLa^{CD73} and SiHa^{CD73} cells proliferation. *, P<0.05.

including cervical cancer. Anti-EGFR therapy has become an effective treatment or for several cancer types (22-24). Accumulating data have showed that HPV activates EGFR/Akt signaling in cervical cancer (25-29). EGFR is present in normal and cervical cancer tumors and is expressed to varying degrees, making cervical cancer more accessible

to targeted therapy. The expression correlation between EGFR and CD73 have been found in several cancer types. For example, Shali *et al.* have reported that CD73 and EGFR expression are positively correlated in liver cancer tissue samples (30). Wu *et al.* have found that CD73 overexpression in colon cancer cell lines can up-regulated

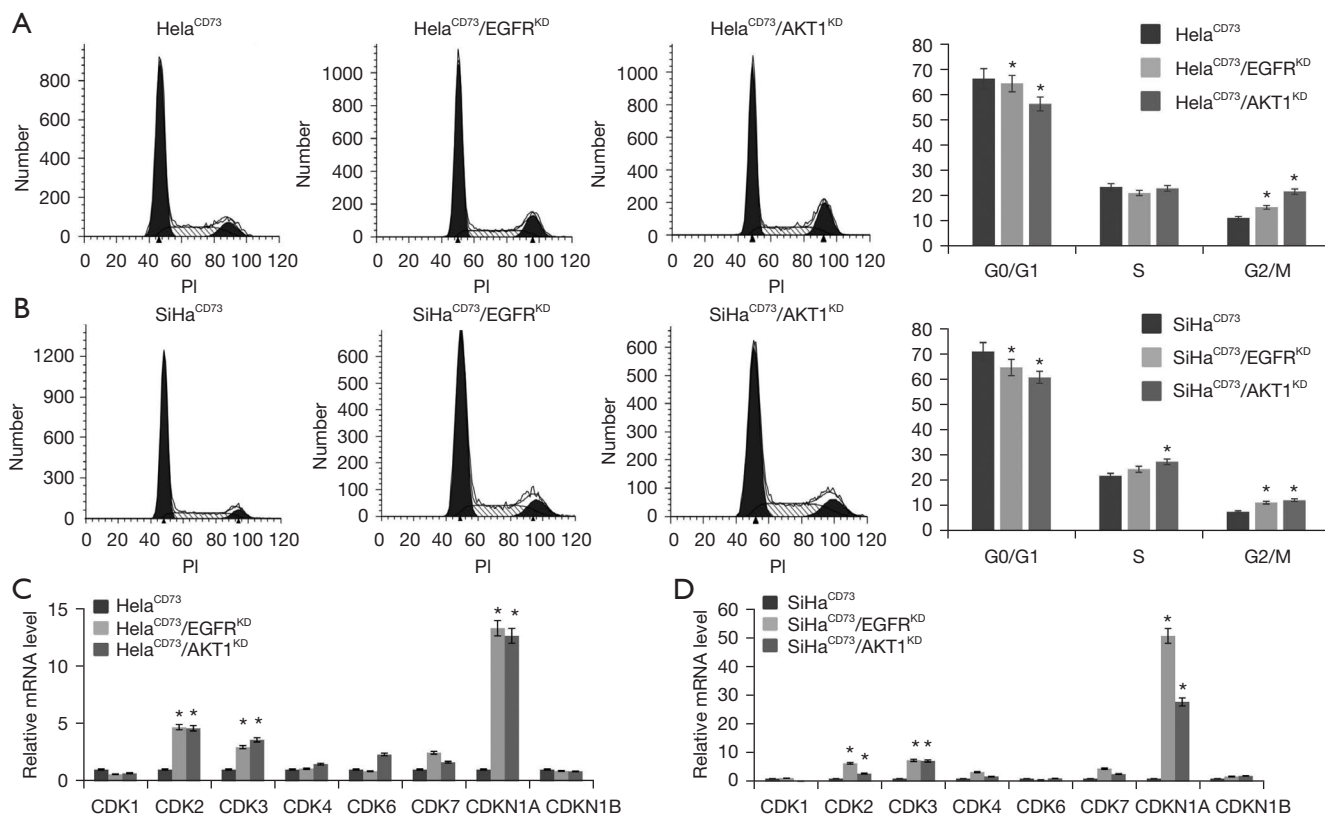


Figure 3 The effects of EGFR and AKT1 knockdown in cell cycles of HeLa^{CD73} and SiHa^{CD73} cells. (A,B) EGFR and AKT1 knockdown result in G2/M phase arrest HeLa^{CD73} and SiHa^{CD73} cells. (C,D) In HeLa^{CD73} and SiHa^{CD73} cells, the expression levels of CDK2, CDK3 and CDKN1A were up-regulated by EGFR and AKT1 knockdown. *, P<0.05.

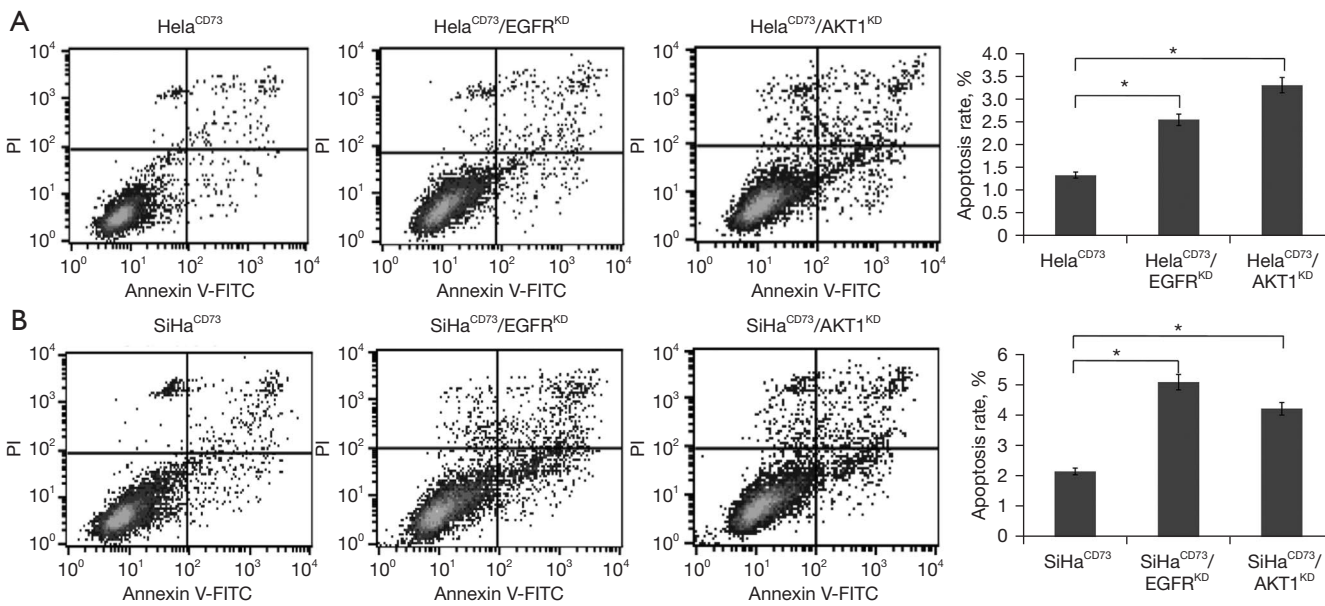


Figure 4 The effects of EGFR and AKT1 knockdown on cell apoptosis. (A,B) EGFR and AKT1 knockdown induced apoptosis of HeLa^{CD73} and SiHa^{CD73} cells. *, P<0.05.

EGFR (31). Our data showed that expression levels of EGFR and AKT1 were increased in cancer cells with higher CD73 levels, and also in transplanted tumor tissues. These results suggested that EGFR and AKT1 played important role in increased growth rate of CD73 overexpression cancer cells. This hypothesis was validation by using EGFR and AKT1 knockdown experiments. Knockdown of EGFR and AKT1 significantly inhibited proliferation of Hela^{CD73} and SiHa^{CD73} via changing cell cycle distribution and inducing cell apoptosis. The mechanism of increasing EGFR/AKT1 levels in CD73 overexpressed cells has not been clarified. Zhi's data have suggested that CD73 may promote EGFR expression in breast cancer through regulating some associated transcription factor, such as PPAR γ (32). Shali *et al.*'s study has suggested that CD73 regulates EGFR through c-Src in hepatocellular carcinoma, which is a critical molecular for EGFR Phosphorylation (30). However, more experimental verification is needed for the potential mechanism between CD73 and EGFR.

CD73 has both enzymatic and non-enzymatic function, not only increase the production of adenosine which leads to immune suppression, but also enhances angiogenesis, tumor growth and metastasis. Recent years, CD73 has been considered as a potential target for cancer therapy. CD73-blockage have showed an anti-tumor effect in animal experiments. Although it has not yet been used clinically, several clinical trials have tried to evaluate the therapeutic effect of CD73 targeting treatment in cancer (15,16). Notably, to overcome cancer complexity, combination of anti-CD73 and -other targets, such as CTLA-4, OX40, PD-L1, etc., would possibility enhance the efficacy of anti-cancer therapy (32,33). Multiple studies with combinatorial target strategies have been reported. Goswami and colleagues have found that the absence of CD73 improved survival in a murine model of glioblastoma multiforme treated with anti-CTLA-4 and anti-PD-1 (33). Sociali have showed that anti-CD73 mAb treatment improves the efficacy of anti-ErbB2 mAb for treating engrafted or spontaneous primary tumors and lung metastases, in mouse models of HER2/ErbB2-driven breast cancer (15). Allard B have found that anti-CD73 mAb significantly enhanced the activity of both anti-CTLA-4 and anti-PD-1 mAbs against MC38-OVA (colon) and RM-1 (prostate) subcutaneous tumors, and established metastatic 4T1.2 breast cancer (14). These studies have presented the potential application value of combined with CD73-blockage in cancer therapy. Although it hasn't been reported yet, our results in this

study suggested the combined of anti-CD73 and anti-EGFR/AKT1 treatment might be a worthwhile research direction.

Notably, it has been well known that CD73-generated adenosine plays an important role in tumor immunoescape. To mediate its immunosuppressive effect, CD73 generated adenosine can bind to G-protein-coupled receptors (i.e., A1R, A2AR, A2BR, A3R) that expressed on cell membrane of immune cells. High concentration of adenosine could suppress immune response via multiple mechanism, such as promoting regulated T cells proliferation, inhibition of M1 macrophage polarization, and blunt the capacity of nature killer (NK) cells (21). Thus, CD73 blockage may also kill tumors by activating immune response *in vivo*.

In this study, we found that EGFR/AKT1 knockdown lead to the upregulation of CDKN1A, and CDK2, and also result to the reduction in G1 phase and increased in G2 phase. Notably, CDK2 was usually considered as a driver of G1 to S phase. Several studies have shown that G2/M arrest was associated with upregulation of CDKN1A (34,35). In this study, the degree of elevated expression of CDKN1A in EGFR/AKT1 knockdown cells was significantly higher than that of CDK2. Thus, we speculated that increased CDK2 trigger G1 to S phase, simultaneous S to G2 phase were triggered with unknown mechanism and upregulation of CDKN1A resulted to increased in G2 phase. Finally, cell cycle distribution was reduced in G1 phase and increased in G2 phase.

There were following limitation of this study. First, although we have try to construct the murine xenograft models by subcutaneous injection of SiHa^{CD73} or SiHa^{NC} cells, while there were no tumor tissue formed, Second, we have found that, EGFR/AKT1 play an important role during the promoted effect of CD73 in cervical cancer growth. However, the mechanism of increasing EGFR/AKT1 levels in CD73 overexpressed cells has not been clarified. The regulated mechanism of CD73 on EGFR/AKT1 pathway should be investigated in future studies.

Conclusions

In conclusion, our data demonstrated that CD73 overexpression promote cervical cancer cell progression *in vitro* and *in vivo*, which was related to increasing of EGFR and AKT1. EGFR and AKT1 knockdown could decrease the CD73 overexpressed cell's proliferation via inducing cell cycle arrest and apoptosis.

Acknowledgments

Funding: This study was supported by the National Natural Science Foundation of China (No. 81702732 to ZWG). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2446/rc>

Data Sharing Statement: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2446/dss>

Peer Review File: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2446/prf>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-21-2446/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. 20200505) granted by Air Force Medical University's Institutional Animal Care and Use Committee, in compliance with Air Force Medical University's guidelines for the care and use of animals.

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Cite this article as: Liu C, Gao ZW, Wang X, Lin F, Zhang HZ, Dong K. CD73 promotes cervical cancer growth via EGFR/AKT1 pathway. *Transl Cancer Res* 2022;11(5):1089-1098. doi: 10.21037/tcr-21-2446