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Regular paper

The cadherin protein CDH19 mediates cervical carcinoma progression by regulating AKT/NF-κB signaling

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The cell adhesion protein cadherin 19 (CDH19) has been reported to be involved in various types of cancer, but its role in cervical carcinoma remains unknown. We collected and analyzed the patients' data using the GEPIA Kaplan-Meier plotter databases. CDH19 was overexpressed in cervical carcinoma cells to assess its effect on cell proliferation and activation of AKT and NF-κB signaling pathways. A xenograft mouse model was established to study the function of CDH19 *in vivo*. We found that CDH19 expression was significantly downregulated in cervical carcinoma tissues compared to adjacent normal tissues. Patients with high expression of CDH19 had a significantly better overall survival rate than those with low CDH19 expression. CDH19 expression was negatively correlated with the expression of the proliferation marker Ki-67, and overexpression of CDH19 significantly inhibited cervical carcinoma cell proliferation. Furthermore, overexpression of CDH19 suppressed the activation of the AKT and NF-κB signaling pathways, and CDH19-overexpressing cervical carcinoma tumors exhibited significantly slower growth *in vivo*. CDH19 plays an important role in cervical carcinoma by suppressing both cell proliferation and the activation of AKT and NF-κB signaling pathways. Therefore, CDH19 may be a potential therapeutic target for cervical carcinoma.

Keywords: CDH19, cervical carcinoma, AKT, NF-κB, nude mice

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Abbreviations: AKT, Protein Kinase B; CCK-8, Cell Counting Kit-8; CDH19, Cadherin 19; CIN, Cervical Intraepithelial Neoplasia; GEPIA, Gene Expression Profiling Interactive Analysis; HPV, human papillomavirus; NF-κB, Nuclear Factor kappa B

INTRODUCTION

Cervical carcinoma is a type of cancer that typically develops in the cervix of women, which is the lower part of the uterus that connects to the vagina. It is caused by the uncontrolled growth of abnormal cells in the cervix. These abnormal cells can spread to other parts of the body and invade nearby tissues and organs. Cervical cancer is one of the most common cancers in women worldwide, with more than 500 000 new cases and over 300 000 deaths reported annually. It is mostly diagnosed in women aged 35 to 44 years, but it can occur in women of any age (Kaur *et al.*, 2003; Lazo, 1999).

The main cause of cervical cancer is the human papillomavirus (HPV), which is sexually transmitted. Cervical cancer often develops slowly over many years, and the early stages of the disease may not cause any noticeable symptoms. However, as cancer progresses, symptoms may include abnormal vaginal bleeding, unusual vaginal discharge, pain during sex, and pelvic pain. But cervical carcinoma can often be successfully treated if detected early (Petereit *et al.*, 1995). Current treatment options for cervical cancer may include surgery, radiation therapy, chemotherapy, or a combination of these approaches, depending on the stage and extent of cancer. However, these treatments might be physically taxing, so it is worthwhile to explore the mechanisms of cervical cancer development can help find new potential therapeutic targets.

Cadherin 19 (CDH19) is a type II classical cadherin protein, which is a cell adhesion molecule that plays an important role in the formation and maintenance of cell-cell contacts. CDH19 protein is expressed in various tissues throughout the body, including the brain, lungs, heart, and kidneys. It is believed to play a crucial role in the development of the nervous system and in the regulation of cell growth and differentiation (Woods *et al.*, 2021). Mutations or alterations in the CDH19 gene have been associated with several different diseases and conditions (Avila & Southard-Smith, 2022; Niu *et al.*, 2008; Tervasmäki *et al.*, 2014). A genetic variation in CDH19 has been linked to a rare neurodevelopmental disorder known as CDH19-related X-linked intellectual disability syndrome. This syndrome is characterized by intellectual disability, seizures, delayed speech and language development, and other neurological and developmental abnormalities. Additionally, CDH19 has been implicated in the developing and progression of certain types of cancer. For example, several studies have reported that CDH19 expression was decreased in cancers including colorectal cancer, gastric cancer, and ovarian cancer. In these cases, reduced CDH19 expression has been associated with poorer outcomes, such as increased tumor invasiveness and poorer survival rates (Blons *et al.*, 2002; Gao & Yang, 2022; Koski *et al.*, 2009; Niu *et al.*, 2008). On the other hand, other studies have reported that CDH19 expression was increased in cancers such as esophageal squamous cell carcinoma and non-small cell lung cancer (Bailis *et al.*, 2019; H. Wang *et al.*, 2021). Overexpression of CDH19 may contribute to cancer growth and metastasis by promoting cell proliferation, invasion, and migration. In these cases, increased CDH19 expression has been associated with more aggressive tumor behavior and poorer prognosis. Overall, the relationship between CDH19 and cancer appears to be complex and may vary depending on the specific type of cancer and other factors. Further research is needed to fully understand the role of CDH19 in cancer development and progression.

Furthermore, there is limited research on the relationship between CDH19 and cervical carcinoma, but some studies have suggested that the expression of CDH19

may be altered in cervical cancer. One study found that CDH19 expression was significantly decreased in cervical cancer tissues compared to normal cervical tissues. Another study reported that CDH19 was upregulated in a group of cervical cancer patients compared to healthy controls (Gu *et al.*, 2021). However, more research is needed to fully understand the role of CDH19 on cervical carcinoma.

The AKT (Protein Kinase B)/NF-κB (Nuclear Factor kappa B) signaling pathway is a crucial pathway involved in cell survival, proliferation, and inflammation. Abnormal activation of this pathway has been implicated in various diseases, including cancer. In cervical carcinoma, studies have shown that the AKT/NF-κB pathway is frequently activated, which leads to the proliferation and survival of cancer cells (Vara *et al.*, 2004). Specifically, activation of the AKT pathway promotes cell survival by inhibiting apoptosis and promoting cell cycle progression, while activation of the NF-κB pathway promotes inflammation and cell proliferation. Moreover, the HPV, which is the primary cause of cervical carcinoma, has been shown to activate the AKT/NF-κB pathway (Bossler *et al.*, 2019). HPV-mediated activation of AKT/NF-κB signaling is thought to contribute to the development and progression of cervical carcinoma by promoting cell survival, proliferation, and inflammation. Overall, the AKT/NF-κB signaling pathway plays a critical role in the development and progression of cervical carcinoma, and targeting this pathway may offer a promising approach for the treatment of this disease. Therefore, our study aimed to explore CDH19's functions and potential therapeutic applications in cervical cancer through AKT/NF-κB signaling pathway.

MATERIALS AND METHODS

GEPIA database and analysis

The expression of CDH19 in cervical carcinoma was collected and analyzed using GEPIA (Gene Expression Profiling Interactive Analysis) database (http://gepia2.cancer-pku.cn). Correlation analysis of gene expression was performed using the Correlation Analysis tool available in the cancer public database GEPIA (http://gepia2.cancer-pku.cn/#correlation).

qPCR

Total RNA was extracted from cells or tissues using a total RNA kit (Invitrogen), followed by the examination of the RNA purity and concentration. The RNA was then reverse transcribed into cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (BioTeke, China). RT-PCR analysis was performed using the SYBR-Green method. GAPDH was used as an endogenous control for normalization. The relative expression levels of RNAs were calculated using the 2-ΔΔCT method. Details of the gene primers used in the study are provided below.
CDH19 F: 5'-CATTTGTAGGCGTGGTGT-3';
CDH19 R: 5'-GTGGCTGTAATACTTAGGTTGT-3';
GAPDH F: 5'-GGT ATCGTGGAAGGACTCATGAC-3';
GAPDH R: 5'-GGT ATCGTGGAAGGACTCATGAC-3'.

KM Plotter database analysis

We analyzed the overall survival of cervical carcinoma patients with high and low levels of CDH19 expression using KM (Kaplan-Meier) Plotter database online (http://kmplot.com) as previously described (Nagy *et al.*, 2021).

Cell culture

The CaSki and C-33A cell lines were purchased from ATCC and cultured as previously described (Liu *et al.*, 2021). Briefly, the frozen cells were thawed rapidly in a water bath at 37°C and transferred immediately to a culture hood where the vial contents were quickly transferred to a sterile tube containing pre-warmed growth medium. Using a hemocytometer or an automated cell counter, the cells were counted to determine the density. Plate or flask choice depended on the experimental requirements and growth characteristics. For routine maintenance, a T25 flask or a 6-well plate was suitable. The cells were seeded and placed in a 37°C incubator with 5% CO_2, creating a controlled environment mimicking physiological conditions. The cells were regularly checked under a microscope to monitor growth and passaged when they reached 70–80% confluency.. To this end, the cells were detached using trypsin-EDTA, next neutralized with the growth medium, and the desired number of cells was transferred to new plates.

Western blot

Protein was extracted from cells or tissues using RIPA lysis buffer, and protein concentration was determined in the lysate. Western blot was performed as previously described (Liu *et al.*, 2012). Briefly, the same amount of protein from different treatment groups were loaded into SDS-PAGE gels, subjected to electrophoresis, and then transferred into PVDF membrane. Then membranes were blocked using 5% milk, then incubated with primary antibody overnight. The primary antibodies against CDH19, *p*-AKT, AKT, GAPDH were purchased from Abcam. GAPDH was used as a loading control.

Cell Counting Kit-8 Assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) assay (Boster, China), following the manufacturer's instructions. In brief, 3×10^3 cells in 100 μL medium were seeded into each well of a 96-well plate, while wells without cells were used as blank controls. Cells in each well were incubated daily with 10 μL of CCK-8 solution for 2 hours at 37°C for 4 consecutive days. Subsequently, absorbance was measured at a wavelength of 450 nm using a microplate reader. The intensity of absorbance is positively correlated with the number of surviving cells (Hou *et al.*, 2022).

Construction and virus packaging of PLVX-CDH19

The full-length human CDH19 gene was amplified via PCR and then inserted into the pLVX-AcGFP lentiviral vector from Thermo Fisher Scientific. Lentiviral particles were produced using a specific protocol that has been previously described (Wang *et al.*, 2017). Cells were transfected with PLVX-NC (negative control) or PLVX-CDH19 for 48 hours.

Nude mouse xenograft model

After transfecting with PLVX-CDH19 for 48 hours, 2×10^6 tumor cells were suspended in 0.2 ml serum-free DMEM and injected subcutaneously into the left flank of each 4-6-week female BALB/c nude mouse. The same number of PLVX-NC-transfected cells was injected into the right flank of each mouse as control. Tu-

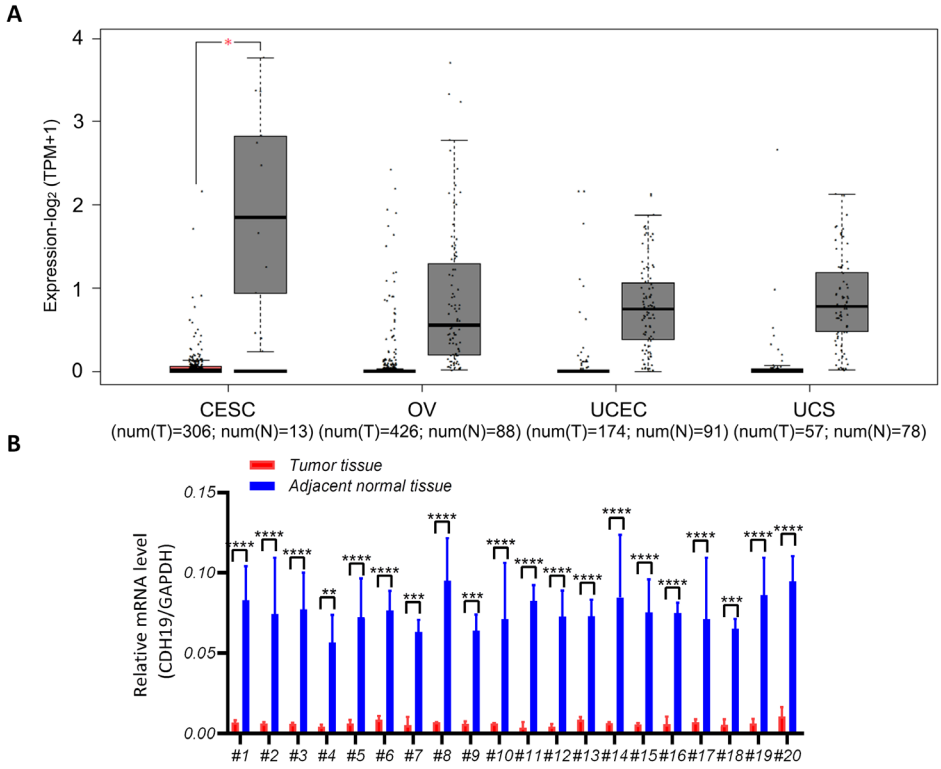

Figure 1. CDH19 is downregulated in cervical carcinoma.
(**A**) The expression of CDH19 in gynecological cancer was analyzed by GEPIA online tool that matches TCGA and GTEx tumor and control transcriptomic data (http://gepia2.cancer-pku.cn). CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; OV, ovarian serous cystadenocarcinoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma. *p<0.01. (**B**) Twenty pairs of cervical carcinoma tumor tissues and paracancerous normal tissues were collected for QPCR analysis against CDH19 and GAPDH. **p<0.01, ***p<0.001, ****p<0.0001.

mor size was recorded every three days. On day 21, the tumors were collected, weighted, and lysed for further analysis as previously described (Liu *et al*., 2020; S. Wang *et al*., 2021). The animal-related protocols were approved by the ethics committee of Zibo Central Hospital. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985).

Statistical analysis

The data was presented as means ± standard deviation, and analyzed with Student's *t*-test, or two-way ANOVA analysis with a post hoc test using GraphPad software. The result was considered statistically significant when the *p*-values were less than 0.05.

RESULTS

CDH19 is downregulated in cervical carcinoma

We used the public cancer database GEPIA to investigate the expression levels of CDH19 in gynecological tumors, including cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC); ovarian serous cystadenocarcinoma (OV); uterine corpus endometrial carcinoma (UCEC)m and uterine carcinosarcoma (UCS) and matched normal tissues The overall expression of CDH19 was low in gynecological tumor tissues, with significant downregulation observed in cervical carcinoma tissues compared to matched normal tissues (p<0.01) (Fig. 1A). To confirm these findings, we collected 20 pairs of cervical carcinoma and adjacent non-cancerous tissues and examined the expression of CDH19. We observed significant downregulation of CDH19 in all 20 cervical carcinoma tumor tissues (Fig. 1B). These results suggest that CDH19 may play a role in the development or progression of cervical carcinoma and could potentially serve as a diagnostic or therapeutic target for this disease.

CDH19 predicts a positive index for cervical carcinoma

Next, we used the public cancer database Kaplan-Meier plotter to perform an online analysis of the overall survival of cervical carcinoma patients with diverse levels of CDH19 expression. As shown in Fig. 2, patients with high CDH19 expression had significantly higher overall

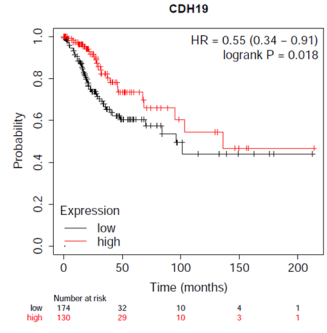

Figure 2. CDH19 predicts a positive index for cervical carcinoma. The overall survival of cervical squamous cell carcinoma with low or high CDH19 was analyzed by Kaplan-Meier Plotter online based on pan-cancer RNA-seq data (http://kmplot.com/).

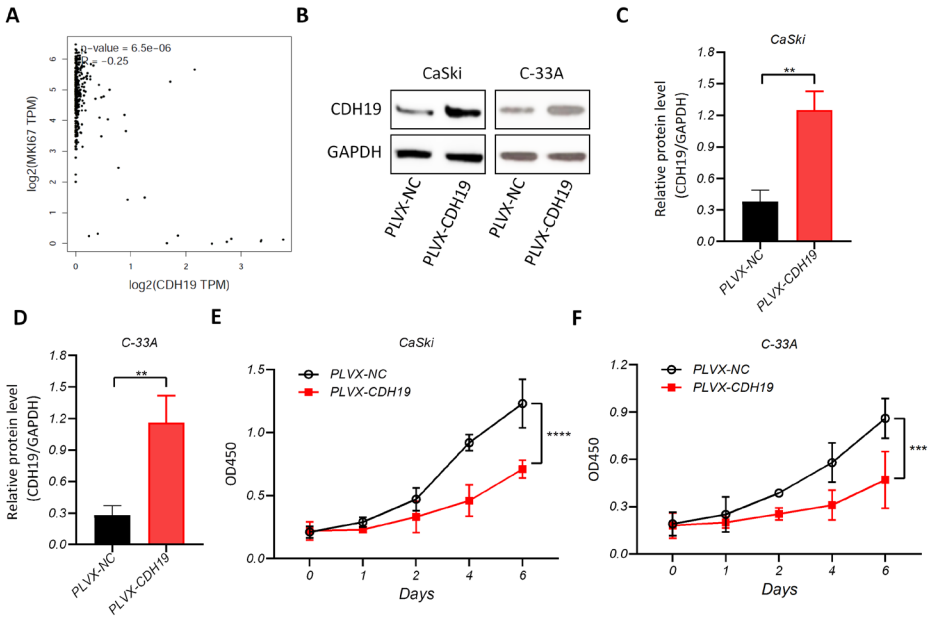

Figure 3. CDH19 overexpression inhibits cervical carcinoma cell proliferation.
(**A**) The correlation analysis between Ki-67 (MKI67) and CDH19 in cervical carcinoma (http://gepia2.cancer-pku.cn). (**B–D**) CaSki and C-33A cells infected with PLVX-NC or PLVX-CDH19-derived lentivirus were lysed for immunoblotting against CDH19 and GAPDH (**B**), and the optical density of the blots was analyzed (**C & D**). E & F. CaSki (**E**) and C-33A (**F**) cells infected with PLVX-NC or PLVX-CDH19-derived lentivirus were prepared for CCK-8 assay at indicated time. ***p*<0.01, ****p*<0.001, *****p*<0.0001.

survival rates than those with low CDH19 expression. These findings suggest that CDH19 expression may be a prognostic biomarker for cervical carcinoma and could potentially be used to stratify patients for more personalized treatment strategies.

CDH19 overexpression inhibits cervical carcinoma cell proliferation

To investigate the potential association between CDH19 and cervical carcinoma cell proliferation, we used the public cancer database GEPIA to analyze the correlation between CDH19 and the proliferation marker Ki-67 in cervical carcinoma tissues. We found a significant negative correlation between CDH19 expression

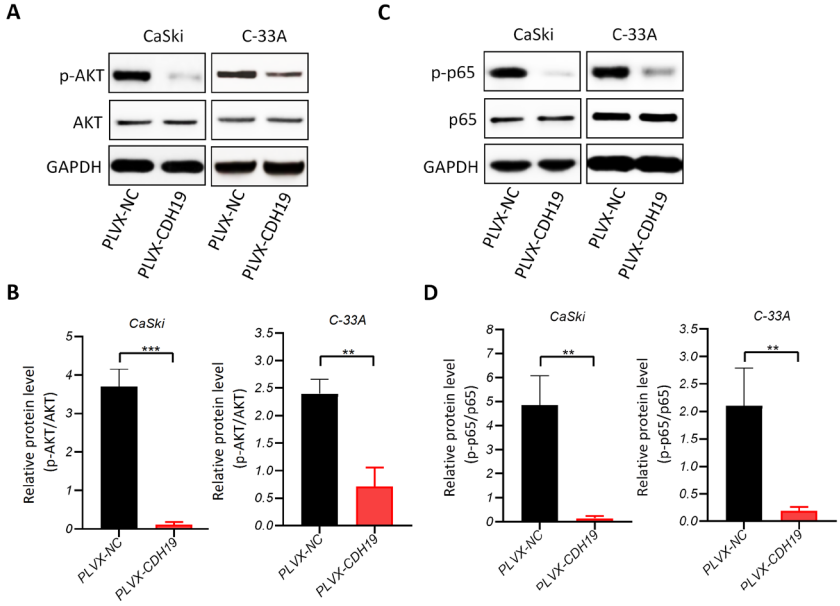

Figure 4. CDH19 overexpression inhibits AKT and NF-κB signaling in cervical carcinoma cells.
(**A & B**) CaSki and C-33A cells were infected with PLVX-NC or PLVX-CDH19-derived lentivirus for 73 hours and then cells were lysed for immunoblotting against p-AKT and AKT (**A**). GAPDH was used as a loading control. The optical density for the blots was also analyzed (**B**). C & D. The above cells were also lysed for immunoblotting against p-p65 and NF-κB p65 (**C**), and the optical density of the immunoblotting was also analyzed (**D**). ***p*<0.01, ****p*<0.001, *****p*<0.0001.

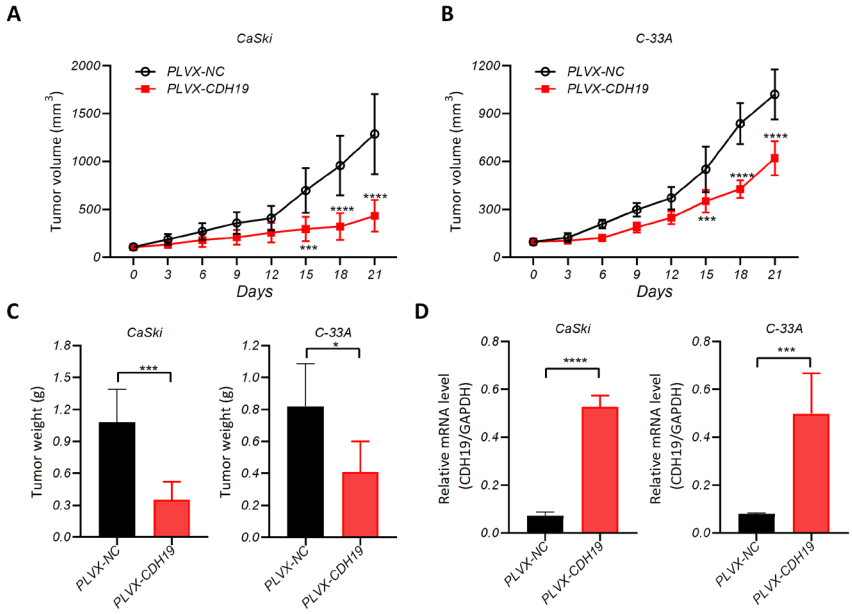

Figure 5. CDH19 overexpression inhibits tumor growth of cervical carcinoma.
(**A & B**) The tumor growth curves. (**C**) At the end of the animal study, tumors were excised and weighed. (**D**) Tumors were lysed for QPCR against CDH19 and GAPDH. *p<0.05, ***p<0.001, ****p<0.0001.

and Ki-67 expression (Fig. 3A). Furthermore, we overexpressed CDH19 in cervical carcinoma cells, which was confirmed by Western blot and PCR (Fig. 3B–D), and observed a significant inhibition of cell proliferation in comparison with negative control (Fig. 3E and F). These findings provide further evidence of a close association between CDH19 and cervical carcinoma cell proliferation, suggesting that CDH19 may play a role in regulating the proliferation of cervical carcinoma cells.

CDH19 overexpression inhibits AKT and NF-κB signaling in cervical carcinoma cells

We further investigated the activity of signaling pathways closely related to cell proliferation and found that overexpression of CDH19 significantly inhibited the activation of the AKT pathway as reflected by the decreased AKT phosphorylation both in CaSki and C-33A cells (Fig. 4A, B). In addition, overexpression of CDH19 significantly inhibited the activation of the NF-κB pathway as evidenced with the decreased phosphorylation of NF-κB p65 subunit (Fig. 4C–D). These findings suggest that CDH19 may be involved in regulating cervical carcinoma cell proliferation through the modulation of AKT and NF-κB signaling pathways.

CDH19 overexpression inhibits tumor growth of cervical carcinoma

Finally, to further validate the functional role of CDH19 in cervical carcinoma, we constructed a xenograft tumor model in nude mice. We found that cervical carcinoma tumors overexpressing CDH19 had significantly slower growth rates *in vivo*, as evidenced by the significantly smaller tumor volume (Fig. 5A, B) and weight (Fig. 5C). Also, CDH19 mRNA expression was significantly upregulated in both CaSki and C-33A cells after transfection (Fig. 5D). These findings provide strong evidence of the inhibitory effect of CDH19 on cervical carcinoma tumor growth and suggest a potential therapeutic target for cervical carcinoma.

DISCUSSION

Cadherins (CDHs) are a family of calcium-dependent cell adhesion molecules that play important roles in the maintenance of tissue structure, and cell-cell communication. CDHs are involved in several key cellular processes, including cell adhesion, migration, proliferation, differentiation, and apoptosis. There are several types of cadherins, including E-cadherin, N-cadherin, P-cadherin, and VE-cadherin. These proteins are expressed in different tissues and play a significant role in embryonic development, tissue morphogenesis, and maintenance of tissue integrity.

Previous studies have shown that alterations in the expression and function of cadherins are associated with various types of cancer In particular, the loss of E-cadherin expression is a common event in many types of epithelial cancers, including breast, gastric, colon, and prostate cancers. E-cadherin is a tumor suppressor protein that helps to maintain cell-cell adhesion, and its loss leads to reduced cell adhesion and increased cell motility, which are important events in cancer metastasis (Li *et al.*, 2020). Additionally, mutations in the genes encoding cadherins have been reported in some cancers, including stomach and breast cancer. For example, mutations in the CDH1 gene, which encodes E-cadherin, have been identified in familial gastric cancer and hereditary diffuse gastric cancer syndrome. These mutations can impair E-cadherin function and lead to an increased risk of developing gastric cancer.

Moreover, changes in the expression and function of other cadherin family members have also been associated with cancer. For instance, N-cadherin is upregulated in many types of cancers and plays a role in promoting cancer cell migration and invasion. Similarly, P-cadherin has been found to be upregulated in certain breast cancers and is associated with a more aggressive tumor phenotype. It is believed that upregulation of N-cadherin and P-cadherin, found in some types of cancer, is associated with a more aggressive tumor phenotype.

In summary, alterations in the expression and function of CDH family members are closely related to the development and progression of cancer. The loss of E-cadherin expression, in particular, is a common event in many types of epithelial cancer and is associated with an increased risk of cancer metastasis (Lechner *et al*., 2013). Understanding the molecular mechanisms underlying these alterations may provide new insights into cancer pathogenesis and may also offer new therapeutic targets for cancer treatment.

CDH19, a member of the cadherin family of cell adhesion molecules, has been implicated in various types of cancers. However, its role in gynecological tumors, especially cervical carcinoma, has not been studied sufficiently (de Mello *et al*., 2017). In this study, we first analyzed the expression of CDH19 in gynecological tumors (CESC, OV, UCEC, and UCS) using the GEPIA database and found that CDH19 expression was overall downregulated in these tumors, including cervical carcinoma.

To further investigate the role of CDH19 in cervical carcinoma, we analyzed the overall survival of cervical carcinoma patients with high or low CDH19 expression levels using the Kaplan-Meier plotter database. We found that patients with high CDH19 expression had significantly better overall survival than those with low CDH19 expression. This data suggested that CDH19 expression was associated with the overall survival rate of cervical carcinoma patients.

Cervical Intraepithelial Neoplasia (CIN) is a grading system used to classify the severity of abnormal cell growth in the cervix. CIN is divided into three grades based on the degree of abnormal cell growth: CIN1 (mild dysplasia), CIN2 (moderate dysplasia), and CIN3 (severe dysplasia or carcinoma in situ). The CIN grading system is widely used in clinical practice to guide management and treatment decisions for patients with abnormal cervical cell growth. In the future, performing the correlation of CDH19 expression with CIN, may better elucidate its role in cervical carcinoma development.

To explore whether CDH19 is associated with cervical carcinoma cell proliferation, we analyzed the correlation between CDH19 and the proliferation marker Ki-67 in cervical carcinoma patient tissues using the GEPIA database. We found that CDH19 expression was negatively correlated with Ki-67 expression. To validate this observation, we overexpressed CDH19 in cervical carcinoma cells and found that CDH19 overexpression significantly inhibited cell proliferation. That means, loss of the CDH19 expression caused abnormal cell growth, and additional CDH19 can significantly inhibit the abnormal growth. These data further give us a hint that CDH19 might be a potential therapeutic target of cervical carcinoma.

We also investigated the activation of signaling pathways that are closely related to cell proliferation and found that CDH19 overexpression significantly inhibited the AKT and NF-$\varkappa$B signaling pathways. There are several studies supporting potential connection between CDH19 and AKT. For example, in prostate cancer, CDH19 has been found to regulate the AKT/mTOR signaling pathway and promote cancer cell migration and invasion (Qian *et al*., 2019). In addition, in breast cancer, CDH19 has been shown to interact with the extracellular matrix protein laminin-511, which activates the AKT signaling pathway and promotes tumor growth (Gudlaugsson *et al*., 2010). These studies suggest that there may be crosstalk between CDH19 and the AKT signaling pathway in various types of cancer. However, so far there are no studies that directly investigate the relationship between CDH19 and the AKT signaling pathway in cervical carcinoma.

Finally, we constructed a xenograft model in nude mice and found that CDH19 overexpression significantly reduced the growth rate of cervical carcinoma tumors *in vivo*. These results further provide evidence that CDH19 might serve as a therapeutic target of cervical carcinoma.

The findings on CDH19 in cervical carcinoma are quite significant in clinic. It not only provides a prognostic marker of cervical carcinoma, but also proposes a therapeutic target. Understanding that CDH19 suppresses the AKT and NF-$\varkappa$B signaling pathways provides crucial insights into the underlying mechanisms of cervical carcinoma. This knowledge could help in developing more targeted and effective therapies that focus on these pathways. Moreover, the *in vivo* studies in a xenograft mouse model validate the potential therapeutic relevance of CDH19. The slower growth of tumors in mice with CDH19-overexpressing cervical carcinoma cells indicates the potential for further exploration in preclinical models, which could eventually lead to human clinical trials. Overall, these findings suggest that CDH19 could have multifaceted clinical implications in the diagnosis, prognosis, and treatment of cervical carcinoma. However, further research and validation are crucial to determine if targeting CDH19 could indeed be a viable approach in clinical settings.

The study shows promising results in cell cultures and xenograft models. However, cellular responses *in vitro* may not always replicate the complexities of human physiology accurately. The behaviors observed in a controlled lab setting might not fully represent the actual response in human patients. Furthermore, this study primarily relies on data collected from databases such as GEPIA and the Kaplan-Meier plotter. While these resources provide valuable insights, they may have inherent limitations, including data quality, biases, or potential errors in the data collection or analysis methods used in these databases. The number and diversity of samples used in the study is limited to 300 (GEPIA dataset) and 400 (TCGA dataset). The findings might not be universally applicable across different demographic or genetic backgrounds. For comprehensive and robust conclusions, a larger and more diverse sample pool should be considered. The findings show correlation between CDH19 expression and patient survival as well as direct influence of CDH19 expression on cell proliferation, and activation of signaling pathways. It's crucial to continue mechanistic studies to further test the cause-effect relationship between CDH19, activation of the cell signaling pathways and patient outcomes to validate the role of CDH19 in cervical carcinoma and to determine its potential as a therapeutic target. While the findings presented in our study are promising, they represent a steppingstone for more in-depth research before any clinical implications can be drawn.

CONCLUSIONS

Taken together, our findings suggest that CDH19 plays an important role in cervical carcinoma, potentially through its regulation of the AKT and NF-$\varkappa$B signaling pathways and inhibition of cell proliferation. Further studies are needed to fully elucidate the underlying mechanisms and potential therapeutic implications of CDH19 in cervical carcinoma.

Declarations

Ethical Approval. The animal-related protocols were approved by the ethics committee of Zibo Central Hospital. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals.

Consent for publication. Current study is available from the corresponding author on reasonable request.

Disclosure of potential conflicts of interest. The authors declare that they have no competing interests.

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Data availability statement. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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