Downregulation of HHLA2 inhibits ovarian cancer progression via the NF-κB signaling pathway and suppresses the expression of CA9

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Abstract

**Background:** HHLA2 has been recently demonstrated to play multifaceted roles in several types of cancers. However, its underlying mechanism in the progression of human ovarian cancer (OC) remains largely unexplored. In the present study, we aimed to determine whether downregulation of HHLA2 inhibited malignant phenotypes of human OC cells and explore its specific mechanism.

**Methods:** In this study, lentivirus was used to establish individual stable cell lines. In vitro tumorigenesis assays, including cell proliferation, invasion, and wound healing assays were performed to evaluate the biological functions of the target genes in ovarian carcinoma cells. Dual-Luciferase assay was performed to identify the CA9 promoter activity. Animal studies were performed to analyze the effect of HHLA2/CA9 on ovarian cancer progression.

**Results:** Downregulation of HHLA2 significantly suppressed the viability, invasion, and migration of OC cells. Downregulation of HHLA2 in OC cells reduced the expression of CA9 and increased the expressions of p-IKKβ and p-RelA. Conversely, the viability, invasion, and migration of HHLA2-depleted OC cells were increased when CA9 was upregulated. In vivo, we found that downregulation of HHLA2 significantly inhibited tumor growth, which was reversed by CA9 overexpression. In addition, downregulation of HHLA2 inhibited the OC progression via activating the NF-κB signaling pathway and decreasing the expression of CA9.

**Conclusion:** Our data suggested a link between HHLA2 and NF-κB axis in the pathogenesis of OC, and these findings might provide valuable insights into the development of novel potential therapeutic targets for OC.

Introduction

Ovarian cancer (OC) is one of the most common gynecologic cancers, and it has the highest mortality rate among malignant tumors of the female reproductive system. OC has few noticeable symptoms in the early stage, and it is prone to distant spread and metastasis. The primary treatment for OC is surgery and adjuvant chemotherapy, while its relapse rate is high, and the 5-year survival rate of OC is only about 20%, which is still a challenge for clinicians and researchers in this field. In recent years, the treatment strategies of OC have been continuously enriched with the improvement of surgical methods, and chemotherapy regimens, and the application of targeted drugs. Improving the treatment approach is vital to reduce the mortality of OC patients. However, although the prognosis of OC has been improved to a certain extent, its overall survival rate is still poor. Therefore, it is urgently necessary to develop novel therapeutics for OC patients.

Maintenance therapy with target drugs has been becoming more widely used in treating OC, and the advancement of immunotherapy is further revolutionizing treatment targets[^1^]. HERV-H LTR-associating 2 (HHLA2), also known as B7H7, B7-H5, or B7y, is a recently discovered member of the B7 family of immune checkpoint molecules[^2^,^3^]. HHLA2 can have either a costimulatory or a coinhibitory effect on T-
cell activation. The costimulatory and coinhibitory effects of HHLA2 on T cells and natural killer (NK) cells are mediated through transmembrane and immunoglobulin domain containing 2 (TMIGD2) and three immunoglobulin domains and long cytoplasmic tail 3 (KIR3DL3), respectively\[^4\]. In addition, HHLA2 is expressed in human cancers, such as breast cancer, lung cancer, thyroid cancer, melanoma, ovary cancer, pancreatic cancer, and cholangiocarcinoma\[^5, 6, 7, 8\]. This protein is found to be localized in both the cell membrane and cytoplasm. The role of HHLA2 on immune cells has been well documented, while previous studies do not agree on the function of HHLA2 in tumor cells. A recent study has revealed that up-regulation of HHLA2 is significantly associated with a favorable outcome for patients with glioma\[^9\]. Another study suggests that aberrant overexpression of HHLA2 activates the JAK/STAT signaling pathway by binding to TMIGD2, thereby promoting immune tolerance in hepatocellular carcinoma cells\[^2\].

The tumor microenvironment (TME) components play critical roles in OC maintenance. Hypoxia is a crucial microenvironment-related factor of solid tumors, including OC\[^10, 11\]. Carbonic anhydrase IX (CA9), a pH-regulating transmembrane protein, is often considered as a surrogate marker of tumor hypoxia and is widely regarded as a prominent biomarker of patients with poor prognosis for many solid cancers, such as kidney cancer and non-small cell lung cancer (NSCLC)\[^12, 13, 14\]. Several studies have demonstrated the critical role of CA9 in tumor growth and metastasis\[^14, 15\]. However, the role of CA9 in OC remains largely undetermined.

As one of the well-studied transcription factor families, NF-κB has been highlighted for its manifold roles in most biological processes, with a particular focus on oncogenesis\[^16, 17\]. Research has shown that Ikkβ-driven NF-κB suppresses malignant progression by preventing the accumulation of reactive oxygen species\[^18\].

In the present study, we clearly showed that downregulation of HHLA2 inhibited the viability, migration, and invasion of OC cells. Moreover, we also investigated whether HHLA2 regulated the expression of CA9 and NF-κB signaling pathways and explored the connection between them.

**Materials And Methods**

**Cell lines and culture conditions**

SKOV3, CAOV3, and ES-2 human OC cells were purchased from the Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences (Shanghai, China). SKOV3 and ES-2 cells were maintained in McCoy’s 5A medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and NaHCO\(_3\) (2.2 g/L) at 37°C in a humidified atmosphere containing 5% CO\(_2\). CAOV3 cells were maintained in DMEM (Invitrogen, USA) supplemented with 10% FBS and sodium pyruvate (100 mM) at 37°C in a humidified atmosphere containing 5% CO\(_2\).

**RNAi lentivirus generation and infection**
Lentivirus was used to establish individual stable cell lines. Briefly, lentiviral particles in cell culture supernatant were harvested at 72 h after lentiviral transduction into 293T cells. The SKOV3, CAOV3, and ES-2 cells were then infected with lentivirus. Finally, stably transfected cells were selected by puromycin (2 μg/mL, Sigma-Aldrich, MO, USA) for 1 week at 48 h after lentiviral infection.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol according to the manufacturer's instructions. qRT-PCR was performed using an SYBR Green Real-Time PCR kit according to the manufacturer's instructions. Primers for the corresponding target genes were as follows: GAPDH forward 5′-GCGGGGCTCTCCAGAACATC-3′ and reverse 5′-TCCACCACTGACACGTTGGC-3′; HHLA2 forward 5′-GGAACACTTCATTTTCCCCAATTTC-3′ and reverse 5′-TCTCTTACATGCTCTCCTTCT-3′, and CA9 forward 5′-GTGTGCAACTGACCCTGCC-3′ and reverse 5′-GGATCTCGGCAGGGAAACGG-3′.

Measurement of the promoter activity

According to the manufacturer's instructions, the CA9 promoter activity was tested using a Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was measured using Single Tube Luminometer (LumiPro).

Western blotting analysis

Cells were lysed in NP-40 lysis buffer (Beyotime) containing 1% phosphatase and protease inhibitors at 4°C for approximately 30 min. Equal amounts of proteins were subjected to SDS-PAGE on 10% gels and transferred onto PVDF membranes. The membranes were blocked with 5% nonfat milk at room temperature for 2 h. Subsequently, the blots were incubated with the following primary antibodies at 4°C overnight, including HHLA2 (Ab214327, Abcam, 1:1,000), CA9 (Ab108351, Abcam, 1:2,000), IKKβ (A19606, Abclonal, 1:3,000), p-IKKβ (AP0369, Abclonal, 1:1,000), RelA (A19653, Abclonal, 1:2,500), p-RelA (AP0123, Abclonal, 1:1000), HRP goat anti-rabbit IgG (AS014, Abclonal, 1:5,000), and HRP-GAPDH (KC-5G5, Aksomics, 1:5,000). Next, the blots were incubated with the appropriate secondary antibody for 2 h. Subsequently, immunoreactive bands were visualized using ECL.

In vitro tumorigenesis assays

Cell proliferation, invasion, and wound healing assays were performed to evaluate the biological functions of the target genes.

For cell proliferation assay, OC cells (2×10^3/well) were seeded into 96-well plates with 100 μL medium, mixed with 10 μL CCK-8 solution (Beyotime, China), and incubated for 2 h. The cell number was counted based on the optical density (OD) value at an optimal wavelength of 450 nm using a microplate reader for four consecutive days. For cell migration assay, matrigel was added into the upper compartment of a 24-well transwell culture chamber, 5×10^5 cells were cultured in 100 μL medium supplemented with 1% serum and seeded into the upper, and 600 μL medium supplemented with 20% serum was added to the
lower compartment. Invasive cells were counted after incubation for 24 h. For wound healing assay, cells were seeded into 6-well plates after growing to confluence, and cross-wound lines were made with a 200-μL pipette tip. Subsequently, the cells were cultured in serum-free DMEM for 24 h, and the wound healing area was recorded. All the experiments were conducted in triplicate.

**In vivo tumorigenesis model**

All mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. For the tumorigenesis model, 8-week-old male BALB/c nude mice were randomly divided into four groups (n=5 per group) with similar weights and maintained under the same feeding conditions. A suspension of $10^7$ Lv-SCramble, Lv-shHHLA2, Lv-SCramble+ CA9, or Lv-shHHLA2+ CA9 group cells in 150 μL of saline was subcutaneously injected into the right flank of each nude mouse (n=5 per group). The tumor volume of mice was monitored every 5 days by the same observer. Mice were euthanized when weight loss was over 20% or when the tumor size was greater than 1,500 mm$^3$. At 30 days after tumor formation, tumors were retrieved and weighed. Tumor volume was calculated as follows: $\text{Volume (mm}^3) = 0.5 \times \text{length} \times \text{width}^2$.

**Immunohistochemistry (IHC) assay**

Paraffin-embedded mouse tumor tissue sections were processed according to standard pathological procedures. The antigen retrieval was conducted by heating the tissue sections at 100°C for 30 min in EDTA solution (1 mM, pH 9.0). The tissue sections were then incubated with primary antibody at 4°C overnight, followed by incubation with HRP-conjugated goat anti-mouse/rabbit secondary antibody. All slides were blindly examined by two independent senior pathologists. The IHC score (0~7) represents a value of staining intensity (0 for no staining, 1 for weak, 2 for clear, and 3 for strong) plus staining average (1 for 0-25%, 2 for 25-50%, 3 for 50-75%, and 4 for 75% above). The antibodies and dilutions were as follows: HHLA2 (Ab214327, Abcam, 1:300), CA9 (Ab108351, Abcam, 1:200), IKKβ (A19606, Abclonal, 1:100), p-IKKβ (AP0369, Abclonal, 1:100), RelA (A19653, Abclonal, 1:100), and p-RelA (AP0123, Abclonal, 1:100).

**Statistical analysis**

Experimental variables were expressed as the mean ± SEM. Statistical analysis was conducted using the paired Student’s t-test. All the statistical analyses were performed using the GraphPad Prism 8.0 software package (GraphPad Software, Inc., San Diego, USA). A $P < 0.05$ was considered statistically significant.

**Results**

**Downregulation of HHLA2 inhibits the cell proliferation, migration, and invasion abilities of OC cells**

We have previously reported that HHLA2 is broadly expressed in OC tissues, which is significantly associated with tumor progression and prognosis, while the underlying molecular mechanisms remain poorly characterized[8]. To analyze the possible role of HHLA2 in malignant phenotypes of OC, three
classic OC-derived cell lines, SKOV3, CAOV3, and ES-2, were employed to evaluate the function and mechanism of HHLA2. We first constructed HHLA2-silencing cells using the above-mentioned three lines (named: Lv-shHHLA2 group cells) and mock control group cells by gene transfection. The qRT-PCR assay demonstrated that a prominently reduced level of HHLA2 was detected in the Lv-shHHLA2 group cells compared with their mock controls (Fig. 1A), this indicated that the knockdown cell line was successfully constructed for subsequent experiments. CCK8 assays demonstrated that Lv-shHHLA2 group cells had a notable decrease in vitality (Fig. 1B). Subsequent wound-healing assay and transwell assay revealed that silencing of HHLA2 markedly inhibited the migration (Fig. 2A, B and C) and invasion (Fig. 2D and E) of Lv-shHHLA2 group cells. Taken together, these findings suggested that HHLA2 silencing inhibits the malignant biological behavior of ovarian cancer cells.

The role of HHLA2 in malignant phenotypes of OC cells is dependent on NF-κB signaling and CA9 in vitro

To further evaluate the molecular mechanism, we determined the changes in the expressions of NF-κB signaling pathway-associated genes that are closely related to malignant tumor progression in Lv-shHHLA2 group cells and their control cells. The Western blotting analysis showed that the expressions of phospho-IKKβ and phospho-RelA were increased after silencing HHLA2, indicating that the NF-κB pathway was activated (Fig. 3E, F, and G). Cancer growth is associated with the development of limited oxygenation (commonly called tumor hypoxia)[19]. CA9 is a member of the carbonic anhydrases' family, that is often expressed in cancer cells under hypoxic condition[20]. We further detected whether the expression level of CA9 in ovarian cancer cells changed after HHLA2 silencing. As shown in Fig. 3A and C, silencing of HHLA2 decreased the expression of CA9 at the mRNA and protein levels in the Lv-shHHLA2 group compared with mock controls. Meanwhile, the luciferase assay also showed that the CA9 promoter activity was significantly reduced (Fig. 3B). In conclusion, we demonstrate that HHLA2 silencing inhibits tumor progression by modulating NF-κB signaling activity and CA9 expression.

Simultaneous downregulation of HHLA2 and overexpression of CA9 promote malignant phenotype of OC cells

To further explore the relationship between HHLA2 and CA9 in the progression of OC, we additionally generated OC cell lines simultaneously overexpressing CA9 and downregulating HHLA2. The results showed that simultaneous downregulation of HHLA2 and overexpression of CA9 failed to decrease the cell vitality (Fig. 4A, B, and C), invasion (Fig. 4D and E), and migration (Fig. 5A, B, and C) of CaOV3, SK-OV-3, and ES-2 cells. Consistently, overexpressing CA9 of OC cells obtained similar results too. The results showed that only the downregulation of HHLA2 decreased the vitality, migration, and invasion of CaOV3, SK-OV-3, and ES-2 cells. These data elucidated that the reduction of CA9 expression caused by the downregulation of HHLA2 suppressed the malignant phenotype of OC cells, while further over-expressing CA9 on HHLA2-depleted cells reversed this suppression.

HHLA2 reduces the expression of CA9 via the NF-κB signaling pathway to inhibit OC progression
Next, we further investigated the role of HHLA2 in the growth of OC cells in vivo via using a nude mouse tumorigenicity assay. Figure 6 shows that the tumors generated from cells transfected with Lv-HHLA2 had a smaller size and weight (Fig. 6A and B) than other groups. Similar to the data from vitro, the tumors generated from cells transfected with Lv-HHLA2 had a smaller size (Fig. 6A) and lower weight (Fig. 6C). We further examined the expressions of CA9, IKKβ, RelA, p-IKKβ, and p-RelA in tumor tissues by IHC assay (Fig. 6D and E). The statistical results showed that downregulation of HHLA2 alone (red) or simultaneous overexpression of CA9 (purple) resulted in increased expressions of p-IKKβ and p-RelA, while HHLA2 downregulation alone inhibited tumor growth due to the reduction of CA9 (Fig. 6F). Therefore, we confirmed that downregulation of HHLA2 reduced the expression of CA9 via activating the NF-κB signaling pathway, thereby suppressing the OC growth.

Discussion

Surgery and chemotherapy are the current primary treatments for OC, while survival rates remain poor[21]. It is urgently necessary to develop more efficient treatment methods. Genes that are crucial to the development of cancer are interfered with targeted molecular therapy to alter cell growth[22]. HHLA2 has been proven to be a potential target for cancer immunotherapy, which is highly expressed in several human cancers[23,24,25]. However, other studies have shown that high expression of HHLA2 predicts a favorable prognosis[5,9,26,27].

In tumor cells, HHLA2 is found in the cytoplasm and membranous regions[3,28]. HHLA2 has two binding receptors, TMIGD2 and KIR3DL3, playing stimulative and inhibitory roles in the immune system, respectively. Our previous study has identified that HHLA2 is overexpressed in OC tissues, which is associated with poor prognosis[8]. In NSCLC, HHLA2 deficiency inhibits malignant phenotype via suppressing EGFR/MAPK/ERK signaling pathway and inhibiting tumor growth in vivo[29]. However, the HHLA2 pathway in OC remains largely unexplored. Therefore, we generated HHLA2-depleted OC cells to explore the effect of HHLA2 downregulation. Cellular proliferation is one of the hallmarks of OC, as well as other cancers in general. Our study revealed that downregulation of HHLA2 inhibited the proliferation of OC cells. The migration of OC cells can result in metastatic lesions that are fatal to patients since they are inoperable. The molecular mechanisms behind the spread of OC are not well understood. We demonstrated that downregulation of HHLA2 inhibited the migration and invasion abilities of OC cells.

Alterations in tumor metabolism and acid/base regulation lead to an acidotic state in the TME that promotes tumor growth and metastasis[10,30,31,32,33]. As a member of the carbonic anhydrase family, CA9 is crucial for controlling the pH inside and outside of the cells and is utilized to identify several metastatic malignancies[13]. Studies have demonstrated that CA9 stimulates the metastatic properties of cancer cells, and downregulation of CA9 suppresses proliferation via metabolic reprogramming and reducing cell migration[14,34]. High expression of CA9 is correlated with significantly reduced survival times of patients[35,36,37]. We demonstrated that downregulation of HHLA2 decreased the expression of CA9 at the mRNA and protein levels. Therefore, the inhibition of malignant phenotype caused by HHLA2
downregulation might be attributed to the decreased expression of CA9. This hypothesis was supported by the fact that CA9 overexpression promoted the malignant phenotype of OC cells, including its proliferation, migration, and invasion, and reversed the inhibitory effects of HHLA2 downregulation.

The various roles that NF-κB activation plays in the inflammatory response are not always pro-inflammatory\cite{38,39,40}. According to previous studies, NF-κB has both pro- and anti-inflammatory functions in inflammation\cite{41,42}. We showed that downregulation of HHLA2 enhanced the phosphorylation levels of IKKβ and RelA, two crucial NF-κB pathway components, and activated NF-κB signaling pathways. Additional in vivo tests supported the findings from the above investigations.

**Conclusions**

In conclusion, our findings demonstrated that downregulation of HHLA2 in OC cells inhibited cell viability, invasion, and migration via the NF-κB signaling pathway and suppressed CA9 expression, whereas overexpression of CA9 might stimulate these malignant phenotypes of OC cells. Taken together, our findings supported a tumor-suppressor effect of HHLA2 downregulation in OC.

**Declarations**

**Ethics approval and consent to participate**

The animal study was reviewed and approved by Animal ethics committee of The Third Affiliated Hospital of Soochow University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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**Author contributions**

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

References


2. Lin Ding, Qian Yu, Shuo Yang, Wen-Jing Yang, Te Liu, Jing-Rong Xian, Tong-Tong Tian, Tong Li, Wei Chen, Bei-Li Wang, Bai-Shen Pan, Jian Zhou, Jia Fan, Xin-Rong Yang, Wei Guo. Comprehensive Analysis of HHLA2 as a Prognostic Biomarker and Its Association With Immune Infiltrates in Hepatocellular Carcinoma[J]. Frontiers In Immunology,2022,13 831101.


25. Pratistha Koirala, Michael E. Roth, Jonathan Gill, Jordan M. Chinai, Michelle R. Ewart, Sajida Piperdi, David S. Geller, Bang H. Hoang, Yekaterina V. Fatakhova, Maya Ghorpade, Xingxing Zang, Richard Gorlick. HHLA2, a member of the B7 family, is expressed in human osteosarcoma and is associated with metastases and worse survival[J]. Scientific Reports,2016,6 31154.


29. Wenjie Sun, Shuying Li, Guiliang Tang, Shaoxing Sun, Yuan Luo, Rui Bai, Linzhi Han, Xueping Jiang, Yanping Gao, Zhengrong Huang, Junhong Zhang, Yan Gong, Conghua Xie. HHLA2 deficiency inhibits non-small cell lung cancer progression and THP-1 macrophage M2 polarization[J]. Cancer Medicine,2021,10 (15):5256-5269.


40. Chao Peng, Yaobin Ouyang, Nonghua Lu, Nianshuang Li. The NF-κB Signaling Pathway, the Microbiota, and Gastrointestinal Tumorogenesis: Recent Advances[J]. Frontiers In Immunology,2020,11 1387.


Figures
Figure 1

Downregulation of HHLA2 inhibits cell growth in OC cells. (A) The expression of HHLA2 at the mRNA level was decreased in Lv-shHHLA2 group cells compared with control cells, measured by qRT-PCR. (B) Downregulation of HHLA2 in SKOV3, CAOV3, and ES-2 cells inhibited cell growth compared with control cells. The data are represented as the means ± SEM; *P < 0.05, **P < 0.01.
Downregulation of HHLA2 inhibits the migration and invasion abilities of OC cells. (A-C) HHLA2 silencing impaired the migration of CAOV3 (A), ES-2 (B), and SKOV3 (C) cells shown by wound-healing assay. Statistical results of the wound-healing assay showed that the migration abilities of Lv-shHHLA2 group cells were significantly decreased. (D) HHLA2 silencing impaired the invasion of SKOV3, CAOV3, and ES-2 cells shown by transwell assay. (E) Statistical results of the transwell assay showed that the invasion
abilities of Lv-shHHLA2 group cells were significantly decreased. The data are represented as the means ± SEM; *P < 0.05, **P < 0.01.

Figure 3

Downregulation of HHLA2 suppresses the expression of CA9 and activates the NF-κB pathway in OC cells. (A) Downregulation of HHLA2 suppressed the expression of CA9 at the mRNA level. (B)
Downregulation of HHLA2 suppressed CA9 promoter activity. (C-D) Downregulation of HHLA2 suppressed the expression of CA9 at the protein level. (E-G) Downregulation of HHLA2 promoted the expressions of p-IKKβ and p-RelA. The data are represented as the means ± SEM; **P < 0.01, ***P < 0.001.
Overexpression of CA9 based on HHLA2 downregulation promotes proliferation and invasion of OC cells. (A-C). Overexpression of CA9 based on HHLA2 downregulation promoted the proliferation of CaOV3 (A), SK-OV-3 (B), and ES-2 (C) cells. (D-E) Overexpression of CA9 based on HHLA2 downregulation promoted the invasion of OC cells. The data are represented as the means ± SEM; *P < 0.05.

Figure 5

Overexpression of CA9 based on HHLA2 downregulation promotes migration of CaOV3 (A), SK-OV-3 (B), and ES-2 (C) cells. The data are represented as the means ± SEM; *P < 0.05, **P < 0.01.
Figure 6

Downregulation of HHLA2 suppresses the tumorigenicity ability of OC in vivo using nude mice xenografts. (A) Representative nude mice xenograft formed by the indicated cells. (B) Statistical analysis of xenograft tumor growth formed by the indicated cells. (C) Tumor weights of the indicated cells. (D) Staining of CA9 and HHLA2 in tumor tissue using IHC. (E) Staining of IKKβ, RelA, p-IKKβ, and p-RelA in
tumor tissue using IHC. (F) Statistical analysis of IHC scores of CA9, HHLA2, IKKβ, RelA, p-IKKβ, and p-RelA.