A cascade via CD276/PI3K/SIRT1/E-Cad in overcoming contact inhibition of proliferation in hepatocellular carcinoma cells

XIAOBO YU[1-3], RONG SU[1-3], GUOQIANG CAO[1-3], HAIYANG XIE[1-3], LIN ZHOU[1-3] and SHUSEN ZHENG[1-4]

[1]NHC Key Laboratory of Combined Multi-organ Transplantation, Key Laboratory of Organ Transplantation, Hangzhou, Zhejiang 310003, P.R. China; [2]Key Laboratory of the Diagnosis and Treatment of Organ Transplantation, Research Unit of Collaborative Diagnosis and Treatment for Hepatobiliary and Pancreatic Cancer, Chinese Academy of Medical Sciences, Hangzhou, Zhejiang 310003, P.R. China; [3]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Hangzhou, Zhejiang 310003, P.R. China; [4]Department of Surgery, Division of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310003, P.R. China

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Abstract. Contact inhibition of proliferation (CIP) halts normal cell proliferation upon confluence, a mechanism often lost in cancer cells, leading to disorganized proliferation. CD276, frequently overexpressed in cancers, promotes proliferation by activating the PI3K/AKT pathway; however, its role in CIP is unexplored. Human HCC cell lines and an immortalized human hepatocyte cell line were cultured under sparse or confluent conditions. Small interfering RNA was transfected to knockdown CD276 expression and plasmid DNA was transfected to overexpress CD276. The cell cycle was analyzed by flow cytometry. Western blotting was used to detect the expression of CD276, E-Cad, SIRT1 and cell cycle proteins. Analysis revealed higher CD276 levels in HCC lines than in HepLi5, with increased CD276 correlating with reduced CIP severity and lower E-Cad expression. Overexpression of CD276 alleviated G0/G1 phase arrest induced by high-density contact, while CD276 knockdown enhanced it. CD276 emerged as a critical regulator of E-Cad in confluent HCC cells through modulating SIRT1 protein via the PI3K/AKT pathway. The findings of the present study highlighted CD276's pivotal role in regulating the cell cycle under confluent culture conditions, revealing a novel regulatory cascade involving CD276/PI3K/SIRT1/E-Cad that may influence tumor progression. This insight into CD276's undisclosed function provides potential treatment strategies for HCC intervention.

Correspondence to: Professor Shusen Zheng, Department of Surgery, Division of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: shusenzheng@zju.edu.cn

Abbreviations: 2D, two-dimensional; 3B, Hep 3B2.1-7; 3B-KD, CD276-knockdown Hep 3B2.1-7 cells; 3D, three-dimensional; 449-OX, CD276-ovexpressing SNU-449 cells; CIP, contact inhibition of proliferation; EMT, epithelial-to-mesenchymal transition; HCC, hepatocellular carcinoma cell; NC, negative control; PBS, phosphate-buffered saline

Key words: CIP, CD276, sirtuin 1, epithelial cadherin

Introduction

Normally, proliferation ceases with a G0/G1 phase arrest of cell cycle, when cells physically contact neighboring cells and upon reaching confluence. This fundamental regulatory process is termed contact inhibition of proliferation (CIP), or simply, contact inhibition (1). This intrinsic control mechanism ensures that cells within a tissue proliferate in a coordinated manner and maintain appropriate spatial organization, prevent uncontrolled cell division and excessive accumulation, which could lead to the formation of disorganized masses or tumors. Cell losses of CIP are more susceptible to malignant transformation and hyperplasia, and such phenomenon has widely been observed in cancer cells (1).

E-Cad and its downstream signaling play a crucial role in regulating CIP (2). The failure to respond appropriately of E-Cad in cancer cells contributes to dysregulation of CIP, while it could be reversed by restoring E-Cad expression (3). Meanwhile, disruption of E-Cad binding between cells using antibodies also reversed CIP (4). Studies have revealed not only E-Cad/catenin adhesion complex, but also E-Cad homophilic ligation are sufficient to regulate CIP (5,6). Despite incomplete understanding of how E-Cad ligation and cytoskeletal tension collaborate to achieve CIP, Hippo pathway, the intracellular signal cascade of E-Cad, has been thoroughly studied (7). Upon activation by E-Cad, YAP undergoes phosphorylation, which retains it in the cytoplasm and thereby suppresses cell proliferation. Inactive Hippo signaling allows YAP to migrate into the nucleus, interact with TEADs, and stimulate target gene transcription and cell proliferation (2).

Numerous oncogenes have been documented to enhance cell proliferation, including notably, CD276. CD276, also known as B7-H3, is a member of the B7 immune checkpoint family of membrane protein and was initially

characterized as a costimulatory molecule on T-cells activation (8). However, CD276 promotes tumor progression either through inducing immunosuppression or non-immunological mechanisms. Non-immunologically, it promotes cancer cell proliferation, invasion and metastasis through activating PI3K/AKT, Jak2/Stat3 and MEK pathway and induces epithelial-to-mesenchymal transition (EMT) via Jak2/Stat3/Slug (9-15). Additionally, it facilitates tumor progression by enhancing tumor resistance to radiotherapy and chemotherapy, augmenting angiogenesis, enhancing aerobic glycolysis, and acting as an active component of tumor-derived exosomes (16,17). With high prevalence of CD276 on malignant cells, inhibitors targeting CD276 are becoming a new class of antineoplastic agents, which show early promising results in solid tumor malignancies (18,19).

SIRT1, an NAD-dependent histone deacetylase, has been implicated in various stages of cancer, particularly playing a pivotal role in regulating cellular proliferation (20). A signal axis of SIRT1-p27KIP was reported to control CIP (21). Recently, Liao *et al* (22) demonstrated that SIRT1 acts as a downstream effector molecule in CD276-induced epithelial-mesenchymal transition process. Considering CD276's established role in facilitating cancer cell proliferation, the present study aimed to elucidate its potential function in enabling cancer cells to overcome CIP, and specifically, whether this mechanism is orchestrated via the activation of SIRT1.

Consequently, it was found that CD276 inversely correlates with E-Cad and positively correlates with overcoming CIP. Increased CD276 reduces G0/G1 arrest under high density by modulating SIRT1 *via* PI3K signaling. This defined a CD276/PI3K/SIRT1/E-Cad pathway critical for hepatocellular carcinoma cells (HCC) to evade CIP.

Materials and methods

Cell culture. Immortalized human hepatocyte cell line (HepLi5) was kindly provided by Prof. Li (23). Human HCC cell lines (SNU-449, HuH-7, Hep 3B2.1-7 and PLC/PRF/5) were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in high-glucose DMEM (cat. no. L100-500; BDBIO; https://www.biocode.cn/product-L100-500) supplemented with 10% fetal calf serum (cat. no. F101-01; Vazyme Biotech Co., Ltd.; https://bio.vazyme.com/product/732.html) and incubated at 37˚C in a humidified chamber containing 5% CO_2.

Cell seeded for spares or confluent culture. The number of cells seeded was calculated based on the total cell count when the monolayer reached 100% confluence for each cell line. For sparse cultures, cells were seeded at 30% of this density, while for confluent cultures, the seeding density was set at 80%. Then, the cells were cultured for ~36 h following seeding and subsequently harvested for processing or analysis.

Cell cycle analysis. Adherent cells were detached using Trypsin-EDTA solution (cat. no. A300-100; BDBIO; https://www.biocode.cn/product-A300-100), rinsed once with phosphate-buffered saline (PBS), and pelleted at 300 x g for 5 min. After supernatant removal, cells were resuspended in 1 ml of PBS. Thereafter, 3 ml of pre-chilled ethanol was added stepwise, and the suspension was stored at -20˚C overnight. The next day, cells underwent centrifugation at 600 x g for 10 min, followed by supernatant disposal. This washing procedure was repeated by adding 1 ml of PBS, vortex, centrifuging, and discarding the supernatant. Afterward, cells were stained with Cell Cycle Staining Buffer (cat. no. CCS012; Multi Sciences Biotech) in darkness for 30 min. Following that, cells were rinsed with PBS, and data acquisition was carried out using a flow cytometer (Canto II; BD Biosciences). Collected data was analyzed using ModFit LT software (version 5.0, Verity Software House, Inc.) for further analysis.

CD276 overexpression. The open reading frame for overexpressing CD276 was generated based on the sequence NM_001024736.2. The coding sequence was cloned into the pcDNA3.1-3xFlag-C vector to generate the overexpression construct. The empty pcDNA3.1-3xFlag-C vector was utilized as a negative control. These molecular cloning procedures were conducted by Guangzhou RiboBio Co., Ltd. On Day 0, a total of $2x10^6$ cells were seeded into a 35 mm dish with 2 ml of serum-supplemented DMEM. Proceeding to Day 1, once cell confluence reached ~50%, 2 μg of plasmid DNA were diluted in 200 μl of jetPRIME buffer (cat. no. 101000046, POLYPLUS-TRANSFECTION S.A; https://shop.sartorius.com.cn/cn/p/jetprime-dnasirna-co-transfection/jetPRIME_DNA_siRNA_co_transfection), followed by vortex and brief centrifugation. Next, 4 μl of jetPRIME reagent were added, vortexed again and centrifuged at 4˚C and 200 g for 30 sec before allowing a 10 min incubation at room temperature to stabilize the mixture. This transfection mixture was subsequently introduced to the cells in serum-containing DMEM and left to incubate for 48 h at 37˚C in a humidified chamber containing 5% CO_2. On Day 3, the cells were harvested for sparse and confluent cultures.

CD276 knockdown. Small interfering RNA (siRNA) sequences were as follows: CD276 siRNA forward, 5'-GUGUGCUGG AGAAAGAUCAdTdT-3' and reverse, 5'-UGAUCUUUCUCC AGCACACdTdT-3'; and negative control forward, 5'-UUC UCCGAACGUGUCACGUdTdT-3' and reverse, 5'-ACGUGA CACGUUCGGAGAAdTdT-3'. siRNAs were synthesized by Guangzhou RiboBio Co., Ltd. On Day 0, a total of $1x10^6$ cells were seeded into a 35 mm dish with 2 ml of serum-supplemented DMEM. Proceeding to Day 1, once cell confluence reached ~50%, 22 pmol of siRNA were diluted in 200 μl of jetPRIME buffer, followed by vortex and centrifuged at 4˚C and 200 g for 30 sec. Next, 4 μl of jetPRIME reagent were added, vortexed again, and centrifuged at 4˚C and 200 g for 30 sec before allowing a 10 min incubation at room temperature to stabilize the mixture. This transfection mixture was subsequently introduced to the cells in serum-containing DMEM and left to incubate for 48 h at 37˚C in a humidified chamber containing 5% CO_2. On Day 3, the cells were harvested for sparse and confluent cultures.

Protein extraction, immunoblotting assay and quantification of protein expression levels. Cell cultures were washed with pre-chilled PBS, then lysed using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). The lysate was incubated on ice for 10 min prior to centrifugation

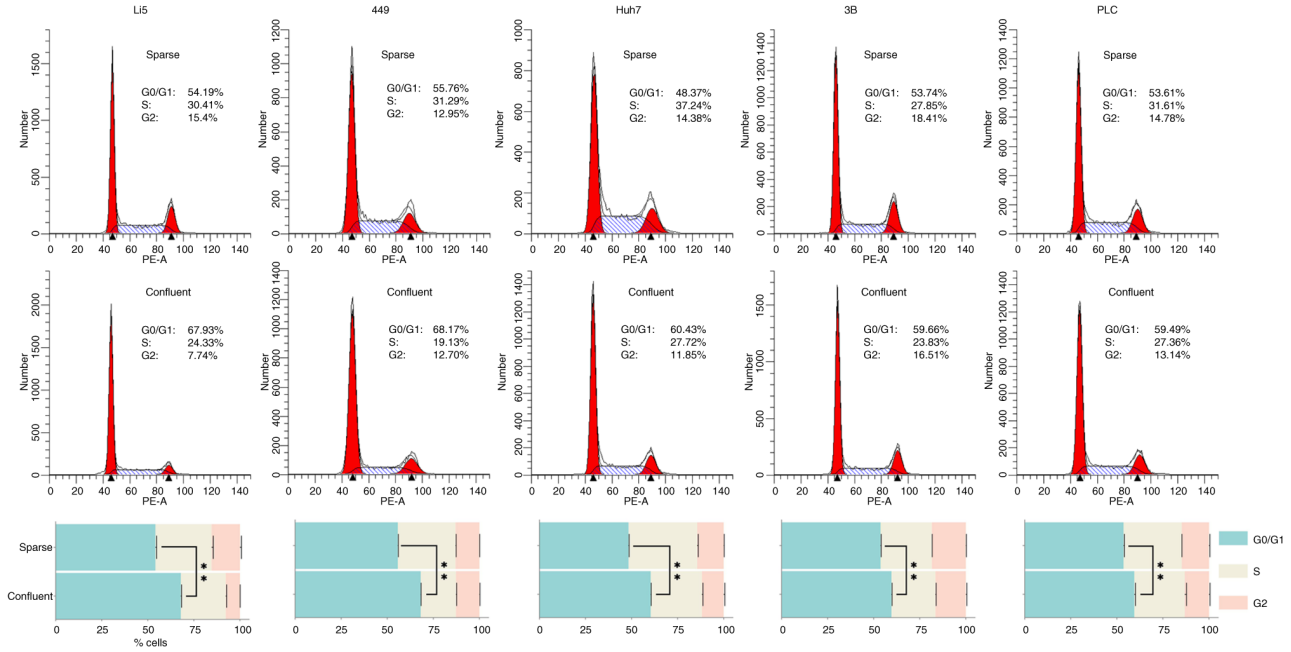

Figure 1. CIP could be observed both in immortalized hepatocyte cells and HCC lines. HepLi5 and the four HCC cell lines (SNU-449, Huh-7, 3B, and PLC/PRF/5) were cultured under confluent conditions, and their cell cycles were analyzed by flow cytometry. The summary data are mean ± SD of three independent experiments. **P<0.01. CIP, contact inhibition of proliferation; Li5, HepLi5; 449, SNU-449; 3B, Hep 3B2.1-7; PLC, PLC/PRF/5.

at 14,000 x g at 4˚C for 15 min. Then the supernatant was collected, and protein concentrations were quantified utilizing a BCA assay kit (cat. no. 23225; Pierce; Thermo Fisher Scientific, Inc.). Equivalent amounts of protein (20 μg per lane) were subjected to 4-12% SurePAGE (cat. no. M00656; GenScript Biotech Corporation) and subsequently transferred to PVDF membranes (cat. no. ISEQ00010; MilliporeSigma) utilizing the eBlot L1 Fast Wet Transfer System (GenScript Biotech Corporation). Membranes were then blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4˚C with primary antibodies: CD276 (1:1,000; cat. no. A17216), E-Cad (1:2,000; cat. no. A24874), p27 (1:1,000; cat. no. A0290), cyclin D1 (1:1,000; cat. no. A11310), GAPDH (1:50,000; cat. no. A19056) and SIRT1 (1:2,000; cat. no. A0230; all from ABclonal Biotech Co., Ltd.). After the primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibody (1:10,000; cat. no. RS0002; ImmunoWay Biotechnology Company) at room temperature for 1 h. Finally, ECL enhanced kit (cat. no. RM00021; ABclonal Biotech Co., Ltd.) was used for detecting protein bounds, and signals were captured by images capturer (ChemiScope, Clinx).

To ensure consistency in protein expression trends, an additional independent biological replicate or cell transfection was performed in duplicate. The resultant data were subsequently employed to validate the previously documented expression patterns (Fig. S1). Protein expression levels were quantified using AlphaView software (ProteinSimple). The loading control proteins were first normalized, followed by calculation of fold change for the target proteins.

Small-molecule inhibitor. The PI3K inhibitor LY294002 (cat. no. HY-10108; MCE; https://www.medchemexpress.cn/LY294002.html) was prepared as a 20 mmol stock solution in dimethyl sulfoxide (DMSO). For further studies, cells were treated with 20 μmol LY294002 for 24 h, while control cells received an equivalent volume of DMSO.

Statistical analysis. Quantitative results are expressed as the mean, accompanied by the standard deviation, calculated from three independent experiments. Statistical analyses were carried out using GraphPad Prism (Version 9.5.0; GraphPad Software, Inc.; Dotmatics). Comparisons between two groups were made using an unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

The magnitude of G0/G1 phase arrest induced by cell-cell contact exhibits variability among HCC lines. Cancer cells are known to be insensitive to CIP; initially, the responses of four HCC lines, SNU-449, HuH-7, Hep 3B2.1-7 and PLC/PRF/5, and an immortalized human hepatocyte cell line (HepLi5) to high-density cell-cell contact were investigated. As expected, confluent HepLi5 exhibited G0/G1 phase arrest with a proportion of cells in G0/G1 phase increased from 54.19 to 67.93%, compared with sparse cultures (Fig. 1). Nonetheless, all 4 HCC cell lines exhibited varying magnitudes of G0/G1 phase arrest as well; specifically, SNU-449 and HuH-7 suffered more severe inhibition (increased from 55.76 to 68.17%, and 48.37 to 60.43% respectively), contrasting with milder extents observed in Hep 3B2.1-7 and PLC/PRF/5 (increased from 53.74 to 59.66%, and 53.61 to 59.49% respectively) (Fig. 1).

CD276 expression coordinates with E-Cad and expression of cell cycle markers. CD276 has been confirmed to promote the proliferation of cancer cells, but whether it plays a role in CIP is currently unclear. Therefore, to investigate this,

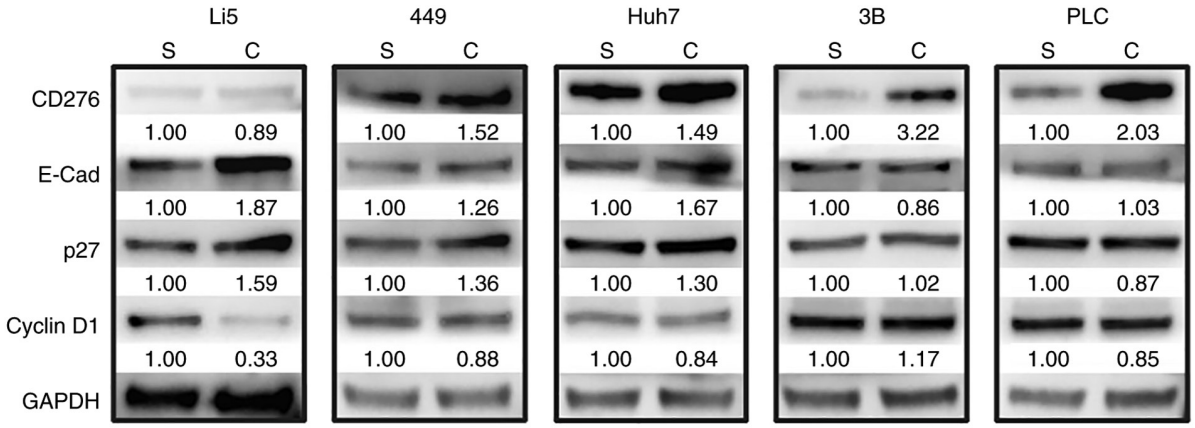

Figure 2. Alterations in the expression of CD276 and cell cycle-related proteins were observed after the occurrence of CIP. The expression of CD276, E-Cad, p27 and cyclin D1 in HepLi5 and the four HCC cell lines (449, Huh-7, Hep 3B2.1-7 and PLC/PRF/5) was analyzed by western blotting. GAPDH was used as a loading control. CIP, contact inhibition of proliferation; 449, SNU-449; 3B, Hep 3B2.1-7; PLC, PLC/PRF/5; S, sparse cultures; C, confluent cultures.

the alterations of CD276 expression within these cells under confluent culture conditions were examined. The expression of CD276 was increased in the four HCC cell lines, whereas no such elevation was found in HepLi5 (Fig. 2). Moreover, CD276 expression demonstrated a more substantial upregulation in Hep 3B2.1-7 and PLC/PRF/5 relative to SNU-449 and Huh-7 (Fig. 2). Intriguingly, cells exhibiting prominent CIP, namely SNU-449, Huh-7 and HepLi5, concurrently displayed notable elevations in E-Cad expression (Fig. 2). Subsequently, alterations of cell cycle-associated markers, such as p27 and cyclin D1, were also found (Fig. 2). These findings suggest that elevated CD276 expression might help cells overcome CIP, possibly through the modulation of E-Cad expression.

Depending on high-density cell-cell contact, CD276 emerges as a critical determinant in regulating E-Cad expression. To further investigate the impact of CD276 on E-Cad expression, cell lines with either CD276 overexpression or knockdown were established. For SNU-449 cells which were sensitive to CIP, CD276 (abbreviated as 449-OX in following context) was overexpressed. While for Hep 3B2.1-7 cells, which were less sensitive to CIP and showed a marked increase in CD276 expression responding to cell-cell contact, CD276 expression [abbreviated as CD276-knockdown Hep 3B2.1-7 cells (3B-KD) in following context] was suppressed. Under sparse culture conditions, CD276 expression was markedly enhanced in 449-OX cells and suppressed in 3B-KD cells, respectively. Under confluent culture conditions, CD276 expression was further increased in 449-OX cells, while it remained markedly suppressed in 3B-KD cells (Fig. 3A). Under sparse culture conditions, the difference in CD276 expression caused only slight changes in E-Cad expression (Fig. 3A). However, under confluent conditions, as the difference in CD276 expression became more pronounced, the difference in E-Cad expression also became more evident, manifesting as suppressed E-Cad expression when CD276 was highly expressed and enhanced E-Cad expression when CD276 expression was reduced (Fig. 3A). These results indicate that CD276 plays a role in regulating E-Cad expression. Similarly, under confluent culture conditions, more pronounced differences in p27 and cyclin D1 expression were observed in confluent 449-OX and 3B-KD cells (Fig. 3A). Consistent with the changes in p27 and cyclin D1 expression, confluent 449-OX cells exhibited a markedly reduced G0/G1 phase arrest compared with control cells; meanwhile, G0/G1 phase arrest was more pronounced in the 3B-KD cells (Fig. 3B). The cell cycle profiles of 449-OX and 3B-KD cells were further studied, revealing no discernible variations in the proportion of cells in G0/G1 phase compared with control cells when cultured under sparse conditions (Fig. 3B). Consequently, the findings of the present study suggested that CD276's regulation of E-Cad expression is highly dependent on high-density cell-cell contact.

SIRT1 is a regulator in the cascade of CD276 mediated E-Cad expression. To ascertain whether CD276 could regulate cell proliferation via SIRT1, the expression of SIRT1 under confluent culture conditions was examined and a downregulation of SIRT1 was observed in HepLi5 cells, whereas no such decline was found in two HCC cell lines, SNU-449 and Hep 3B2.1-7 (Fig. 4A). Subsequently, in 449-OX cells, an elevation was found in SIRT1 expression when cultured under confluent conditions (Fig. 4B); by contrast, in 3B-KD cells, SIRT1 expression decreased (Fig. 4C), with its expression change being consistent with that of CD276 (Figs. 4B and 3A). Moreover, when LY294002, an inhibitor of PI3K pathway, was added, the increase in SIRT1 expression in 449-OX cells was abolished, while LY294002 did not significantly affect CD276 expression (Fig. 4B). These findings strongly suggested that CD276 indeed exerts a regulatory effect on SIRT1, with the modulation occurring via the PI3K/AKT pathway.

Discussion

The present study investigated the role of CD276 in proliferation inhibition induced by high-density cell-cell contact, elucidating that CD276 modulates SIRT1 *via* the PI3K/AKT pathway, leading to suppressed E-Cad expression and thereby partially alleviating CIP. Previous studies have established CD276's capability to enhance cellular proliferation through the PI3K/AKT, Jak2/Stat3, and MEK pathways (9-11), while the present study represented the first documentation of CD276's function in enabling cancer cells to maintain their proliferative potential by suppressing E-Cad during CIP.

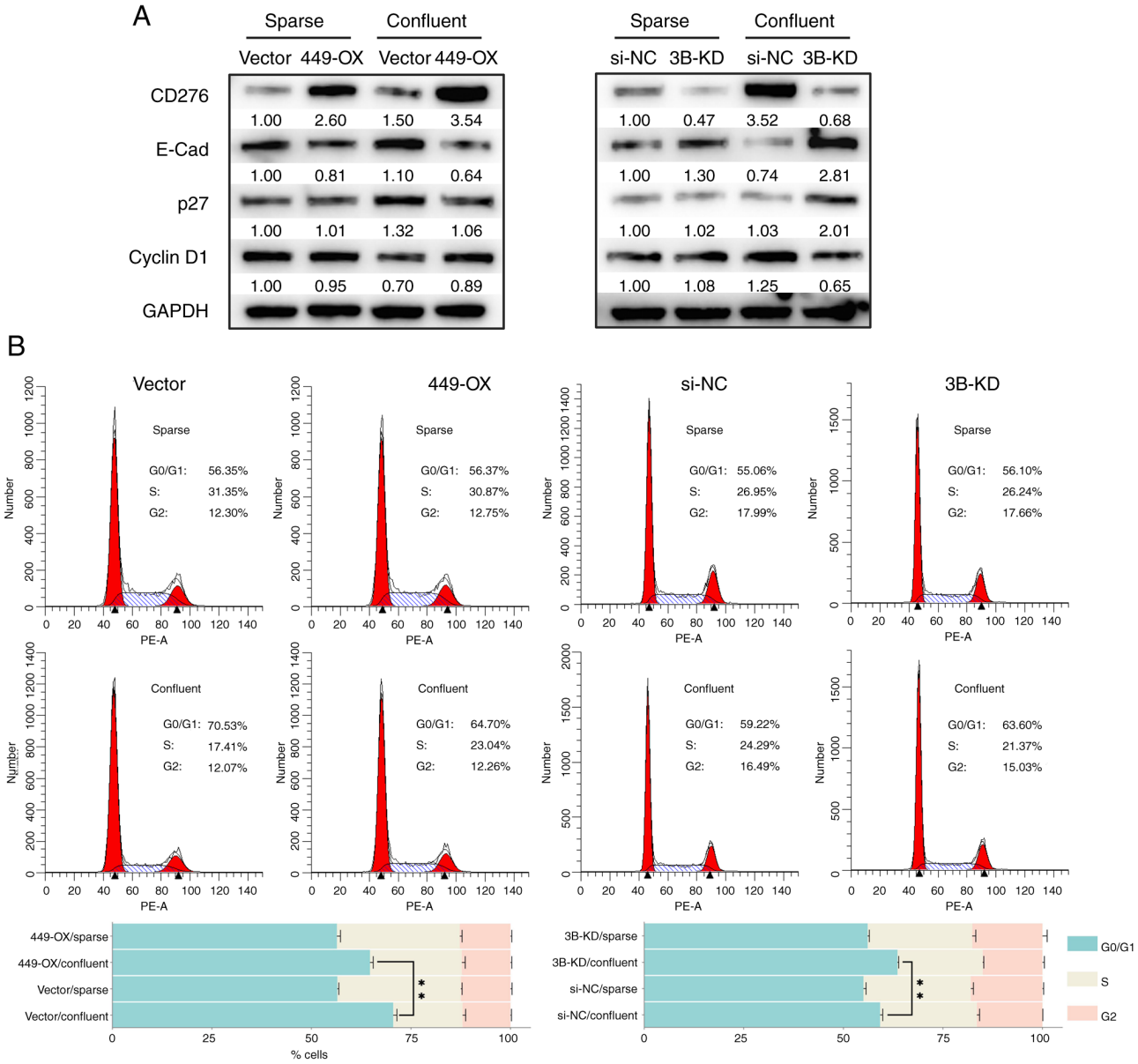

Figure 3. Overexpression or knockdown of CD276 significantly affected E-Cad expression and cell cycle arrest, which is highly dependent on high-density cell-cell contact. 449-OX cells and 3B-KD cells were cultured under sparse or confluent conditions. (A) The expression of CD276, E-Cad, p27 and cyclin D1 was analyzed by western blotting, and (B) their cell cycles were analyzed by flow cytometry. **P<0.01. 449-OX, CD276-ovexpressing SNU-449 cells; 3B-KD, CD276-knockdown Hep 3B2.1-7 cells; si-NC, small interfering-negative control.

Initially recognized as a member of the immune checkpoint proteins, CD276 plays a pivotal role in regulating the immune system, crucial for both immune tolerance and recognition (18). Its ubiquitous expression across various cancer cell types facilitates the evasion of these cells from cytotoxic T-cell and natural killer cell surveillance (24). Over time, accumulating evidence has shed light on CD276's involvement in tumor proliferation, metastasis, treatment resistance and poor prognosis (17). More recently, studies have shown the existence of a CD276/SIRT1 axis in cancer (22,25), which either promotes EMT or suppresses growth arrest. Both the investigations consistently revealed that CD276 modulates SIRT1 through AKT phosphorylation. Previously, Cho and Dai (21) confirmed that SIRT1 regulates E-Cad expression, thereby influencing CIP. Given these precedents and upon discovering CD276's potential involvement in CIP and its capacity to regulate E-Cad, the authors were enlightened to consider SIRT1's role, as a downstream factor of CD276, in this process. Consequently, the investigation of the present study successfully delineated a regulatory pathway involving CD276/PI3K/SIRT1/E-Cad.

Considering the pivotal roles of CD276 in inducing immune tolerance, facilitating immune evasion, and promoting tumor proliferation and migration, therapeutic interventions targeting CD276 have garnered significant interest. To date, numerous clinical programs investigating CD276 inhibitors are currently underway, including enoblituzumab, omburtamab, HS-20093, ifinatamab deruxtecan and vobramitamab, all of which have consistently demonstrated varying degrees of antitumor activity (17,19). The therapeutic mechanisms of these agents are primarily based on either the immunosuppressive function of CD276 or its pan-cancer expression pattern. However, the

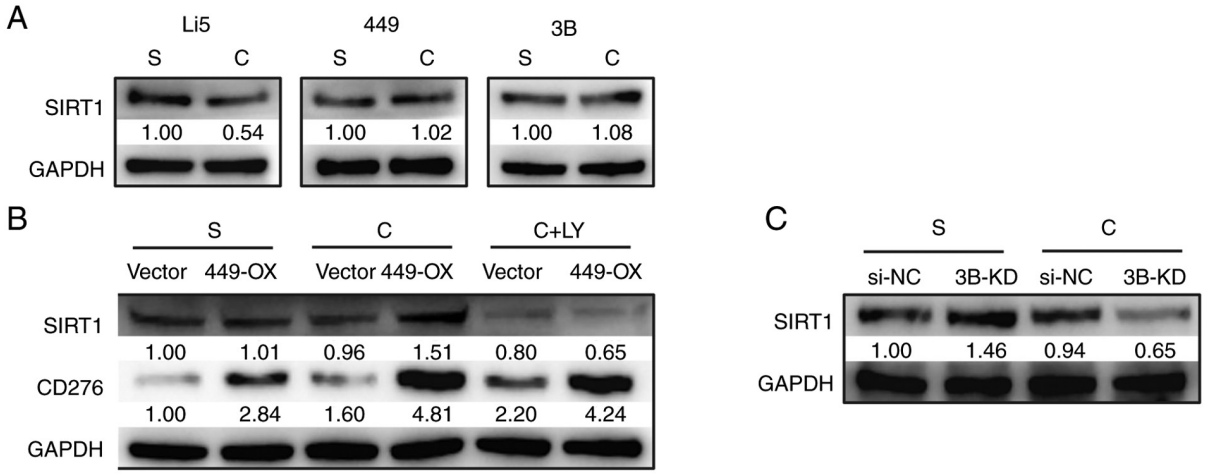

Figure 4. SIRT1 acts as a downstream regulator of CD276 in mediating E-Cad. (A) The expression of SIRT1 was analyzed by western blotting in HepLi5, SNU-449 and 3B cell lines under sparse or confluent conditions. (B) The expression of SIRT1 in 449-OX cells was analyzed by western blotting under three conditions: sparse, confluent, and confluent with additional treatment of LY294002. (C) The expression of SIRT1 was analyzed by western blotting in 3B-KD cells under sparse and confluent conditions. 3B, Hep 3B2.1-7; 449-OX, CD276-ovexpressing SNU-449 cells; Li5, HepLi5; 449, SNU-449; 3B, Hep 3B2.1-7; S, sparse cultures; C, confluent cultures; LY, LY294002; si-NC, small interfering-negative control; 3B-KD, CD276-knockdown Hep 3B2.1-7 cells.

present research on the role of CD276 in CIP suggests that CD276 likely functions within the core or central region of tumor masses, areas characterized by limited blood supply. This implies that monoclonal antibodies and antibody-drug conjugates currently in Phase 1/2 clinical trials may not efficiently penetrate and act on the interior of such tumor cell clusters. Nevertheless, developing potent small-molecule inhibitors targeting CD276, especially when combined with targeted nanoparticle delivery systems, could overcome this limitation. Such an approach may inhibit tumor proliferation at an earlier stage and holds significant promise for application in neoadjuvant therapy.

The cascade that was validated in the present study, involving CD276/PI3K/SIRT1/E-Cad, not only enriches the author's comprehension of CD276's multifaceted functions but also expands the potential applications of CD276-targeted therapies in cancer treatment, suggesting new avenues for intervention in tumor progression and potentially augmenting the effectiveness of immunotherapies. In solid tumors, the proliferation of tumor cells is accompanied by their ability to breach CIP imposed by cell-cell interactions, ultimately leading to insensitivity to contact inhibition in their proliferation. Tumor cells not only overcome CIP but also evade contact inhibition of locomotion (CIL), enabling them to leave the primary site and migrate (26). Given the ubiquitous nature of this phenomenon across solid tumors, its regulatory mechanisms are likely highly conserved. Therefore, the signaling axis mediated by CD276 may also operate in other solid tumor types. The present study is the first to identify the role of the CD276/E-Cad axis and its downstream pathways in this process, providing new insights into the pathogenesis and treatment of solid tumors.

In vitro tumor research based on two-dimensional (2D) cell culture offers advantages such as operational simplicity and low cost. Due to the absence of nutrient heterogeneity, this system often provides high experimental reproducibility. In the present study, a 2D, anchorage-dependent culture system, was utilized. However, such a system might have inherent limitations in the present work, notably the inadequate cell junction formation and insufficient response to mechanical cues (27,28), leading to physiological and cellular response that differ from those observed *in vivo*. Extensive research has underscored the critical role of mechanical transduction in CIL and CIP (26). Moreover, it poorly mimics tumor growth in 3D settings, as it assumes spatial uniformity among cells, making it difficult to accurately study internal tumor cell distribution and growth dynamics. Consequently, numerous studies have shifted towards 3D culture models to better investigate cell-cell contact, yielding substantial insights (21,29,30). These 3D systems partially mimic key aspects of solid tumors more closely, such as structural complexity, central hypoxia and gradients of oxygen and nutrients. Notably, 3D cultures enable cells to receive contact stimuli from multiple orientations, thereby providing a more accurate reflection of *in vivo* conditions than 2D monolayer cultures and serving as a more realistic model for studying contact inhibition (31). Despite inherent limitations, 2D culture in the present study successfully unraveled CD276's role in cell-cell contact inhibition. It was hypothesized that the efficacy of the 2D culture system may stem from CD276's early engagement in contact inhibition processes, which might be highly sensitive to contact and occurs at the moment cells just make contact, thus becoming observable within 36 h in the present study. Recently, Sutton *et al* (32) reported that membrane-localized 4Ig-CD276 enhances cancer cell proliferation through a dimerization-dependent intrinsic signaling. Insightfully, it is plausible that high-density cell-cell contact fosters 4Ig-CD276 dimerization, triggering downstream activation, although mere overexpression of CD276 alone may not elicit substantial effects. Hence, while 3D cultures often provide a more intricate simulation of *in vivo* conditions, the results of the present study emphasized the utility of 2D models for capturing early molecular events mediated by CD276 in contact inhibition, potentially due to its swift response to initial cell contacts and the need for specific structural configurations, such as dimerization, to full regulatory functionality.

In vitro cell experiments often involve prolonged culture durations, which can culminate in elevated cell densities at the time of analysis, possibly activating contact inhibition-related proteins and introducing bias into the outcomes. A recent study on CD276's function in promoting EMT in esophageal squamous cell carcinoma (33) revealed enhanced E-Cad expression in CD276 knockdown cells; conversely, suppression was observed in cells overexpressing CD276. This observation differs slightly from the results of the present study and might be attributed to a lower/higher cell density in the culture system during protein harvest. Therefore, meticulous management of cell density throughout experiments is crucial in studies of cell cycle to ensure the validity of interpretations and minimize artifacts attributable to contact inhibition.

In summary, employing a 2D culture system, a regulatory pathway involving CD276/PI3K/SIRT1/E-Cad that governs CIP was elucidated, thereby expanding the potential applications of CD276-targeted therapies in cancer treatment. The findings of the present study also indicated that CD276's involvement in cell cycle regulation is contingent upon high-density cell-cell contact, highlighting the importance of considering cellular context when examining its function in proliferation control.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XY performed experiments, validated data and wrote the original draft. RS conducted statistical analysis. GC managed and maintained the research data. HX supervised the study. LZ conceived the study. SZ conceived and supervised the study. All authors read and approved the final version of the manuscript. XY and SZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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