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Original Article

Overexpression of gasdermin D promotes invasion of adenoid cystic carcinoma

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Abstract: Objective: To investigate the relationship between gasdermin D (GSDMD) expression and the invasion of adenoid cystic carcinoma (ACC). Methods: Immunohistochemistry (IHC) was used to examine GSDMD expression in tumours and adjacent non-cancerous (ANC) tissues from 33 patients with salivary ACC patients and in tumour samples from 29 patients with pleomorphic adenoma (PA). Lentiviral infection was used to stably overexpress GSDMD in ACC-LM and ACC-83 cells (GSDMD-ov cells), which were subjected to transwell and scratch tests to assess their invasive abilities compared to control cells. Cells overexpressing GSDMD were treated with siRNA targeting GSDMD, and their invasive ability was subsequently examined. Results: GSDMD expression was significantly higher in ACC tissues than in corresponding ANC tissues ($P<0.001$). After 24 hours, both the ACC-83 and ACC-LM GSDMD-ov cell lines had more cells that moved through the membrane than did the control cells ($P<0.05$). For the wound healing experiment, the diameter of the wound in the GSDMD-ov cell lines was smaller than that of the control cells ($P<0.001$) after 24 hours. The ACC cell lines expressing high GSDMD showed stronger metastatic ability than did the control. Conclusion: GSDMD was highly expressed in ACC tissues compared to ANC tissues, and high GSDMD expression promoted the invasion of ACC cells. These findings suggest that GSDMD expression is related to the invasion of ACC. Our data indicate that we may be able to use GSDMD as an indicator of the invasive or metastatic potential of tumour cells in future research.

Keywords: Adenoid cystic carcinoma, GSDMD, pyroptosis, invasion

Introduction

Salivary gland tumours account for 3% of all cancer [1]. Epidemiological studies have indicated that salivary adenoid cystic carcinoma (ACC) is one of the most common types of salivary gland cancer in China [2], accounting for 11% of epithelial tumours and 27% of malignant salivary tumours [3, 4]. Surgery in conjunction with radiotherapy and chemotherapy has been performed in the treatment of ACC over the years, with positive outcomes [5, 6]. Invasion and metastasis are malignant processes of ACC [7], and ACC is highly prone to perineural invasion and easily invades blood vessels to cause metastasis [8]. However, the outcome for ACC is not satisfactory [9, 10]; therefore, identifying specific markers that are closely related to the biologic characteristics of tumours is urgently needed to predict the outcome of ACC and the performance of an antigen-specific therapeutic targeting strategy.

Gasdermin D (GSDMD) is a member of the gasdermin family of proteins [11, 12]. GSDMD was recently identified as a factor responsible for the inflammatory form of lytic cell death, pyroptosis, a critical innate antibacterial immune defense mechanism [13-15]. As a newly identified form of programmed cell death, pyroptosis is involved in various diseases and may affect tumour patients [16, 17]. Previous studies generally focused on the pyroptosis process, which has been reported to be involved in tumour progression, invasion, and metastasis in many different tumour types [18, 19]. However, whether GSDMD, the key effector protein of pyroptosis, is directly involved in promoting tumour invasion remains unclear, as no related research exists to date.

In this paper, we report that GSDMD expression was higher in ACC than in adjacent non-cancerous (ANC) tissues. However, whether GSDMD affects invasion in ACC remains unknown;

hence, the purpose of our study was to determine whether GSDMD affects the invasive ability of ACC.

Materials and methods

Immunohistochemistry and evaluation

Thirty-three matched pairs of ACC and ANC tissues from patients as well as 29 samples of pleomorphic adenoma (PA) tissues were collected from 2011 to 2016 at the Department of Oral and Maxillofacial Surgery, Sun Yat-Sen Memorial Hospital. ANC tissue refers to an area at least 2 cm away from the tumour lesion representing the border of resection, which is called the safe resection margin, and the ANC tissues were pathologically verified [20]. The specimen diagnosis was confirmed by a histopathologic examination. No patient had received any prior therapy, such as radio- or chemotherapy. All tumours were histologically classified according to the World Health Organization classification and staged according to the guidelines of the Union for International Cancer Control [21, 22].

All tumours were fixed in 10% formalin buffer and dehydrated before they were embedded in paraffin. The blocks were sliced into 5-μm sections. Sections for immunohistochemistry (IHC) were retrieved by heating the samples in citrate buffer in a microwave. Hydrogen peroxide (3%) was used to block endogenous peroxidase activity, and serum was used to block non-specific antigens. GSDMD polyclonal antibody (1: 800, Proteintech, USA) was incubated with the samples for 60 minutes at 37°C. After incubation with streptavidin HRP (PV-6000, Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China), the sections were developed with 3,3'-diaminobenzidine and counterstained with haematoxylin.

Immunohistochemical staining was evaluated by 2 experienced pathologists who were blinded to the clinicopathologic data [23]. The tumour was recorded as positive if more than 10% of the tumour cells showed positive staining. According to the staining intensity, tumours were classified as negative, weakly positive and positive. Oral mucosal squamous epithelium was used as a positive control for GSDMD expression, and normal salivary tissues were used as a negative control.

Cell line and cell culture

The human adenoid cystic carcinoma cell lines ACC-LM and ACC-83 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). We routinely cultured the cells at 37°C in a humidified incubator containing 5% CO_2. RPMI 1640 medium containing 10% fetal bovine serum was used.

Transfection for stable expression

HEK293T cells were seeded into a 10-cm plate and cultured with DMEM supplemented with 10% FBS in a 37°C humidified incubator containing 5% CO_2. The cells were then transfected with 6 μg of psPAX2, 3 μg of pMD2.G and 10 μg of transfer vector using Lipofectamine 3000 reagent according to the manufacturer's instructions. At 48 hours after transfection, the medium was collected and used to transfect ACC cells for 48 hours. Puromycin-resistant ACC cells were selected, and verification of the stable cell lines was conducted using western blot and RT-PCR. Cells transduced with empty vector were used as a control.

Small interfering RNA (siRNA) transfection

siRNAs targeting GSDMD were used as follows: Negative control siRNA was purchased from Gemma Pharma Biotechnology (Suzhou, China). Steps were taken to determine the efficiency of the experiments. We performed siRNA transfection with Lipofectamine 3000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The experimental group was transfected with a siRNA sequence targeting GSDMD, and the control group was transfected with a non-targeting siRNA sequence.

RNA extraction and real-time quantitative RT-PCR

At 24 hours after transfection was completed, total RNA was extracted using TRIzol (TaKaRa, Japan) according to the manufacturer's instructions. The Bestar qPCR RT Kit (DBI Bestar® SybrGreen) was used to transcribe the RNA into cDNA according to the corresponding instructions. Then, GSDMD expression was detected using the cDNA as a template.

In brief, 1 μl of cDNA was mixed into a 20 μl reaction mixture according to the instructions of the qPCR PrimeScript™ RT Master Mix

(Takara) and three replicates per condition were used. The reaction was then carried out using the TB Green® Premix Ex Taq™ II (Takara) system with the following cycling conditions: 94°C for 2 minutes and 40 cycles of 94°C for 20 seconds, 58°C for 20 seconds and 72°C for 20 seconds. Each experiment was performed three times with each experiment containing three replicates; the average of the three replicates was used as a single data replicate. GAPDH expression was used as a reference to standardize GSDMD expression. The relative mRNA expression was detected using LightCycler 480 II Real-time PCR machine (Roche, USA).

Protein extraction and western blotting analysis

Western blot analysis was used to confirm the effects of the transfected plasmid on GSDMD expression in the experimental and control groups.

The cell culture medium was removed, the cells washed twice with ice-cold phosphate-buffered saline, and an appropriate amount of lysis buffer (Pierce™, No. 23227) was added and incubated with the adherent cells at 4°C for 30 minutes. Next, the cells were scraped, the lysate was collected and centrifuged, and the supernatant was collected. The protein concentration was determined using a BCA Protein Assay Kit B according to the manufacturer's instructions.

Western blotting was performed using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime, Shanghai, China) with 20 μl of protein solution (concentration: 1 μg/μl) loaded per lane. Upon completion, the proteins were transferred to Immobilon-P Transfer Film (PVDF) (Beyotime, Shanghai, China), which were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 on a shaker at room temperature. Primary antibody (1:1000 in 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20) against GSDMD (Abgent, Suzhou, China) was added to the membrane and incubated overnight on a shaker at 4°C. The membranes were then washed with Tris-buffered saline containing 0.1% Tween-20 and probed with secondary antibody (anti-rabbit; 1:2000; Shanghai ExCell Biology, Inc., China). Primary antibody (1:1000) against GAPDH (KangCheng-Biotech, Shanghai, China) was used as a control for protein loading. The western blot analysis process was performed according to standard protocols.

In vitro invasion assay and migration assay

We used a polycarbonate Transwell plate for the invasion assay. Specifically, 2×10^5 cells in RPMI 1640 medium containing 1% FBS were seeded into the upper chamber of a polycarbonate Transwell plate (8 μm pore size; Corning, USA). The lower chamber contained RPMI-1640 medium with 15% FBS as the chemoattractant. After the plates were incubated for 24 hours in a 37°C incubator, they were removed. The medium was removed, and cells on the surface of the upper chamber that did not pass through the membrane were removed a cotton swab. Cells on the surface of the lower chamber were fixed in methanol at 4°C for 20 minutes and then stained with 0.1% crystal violet for 20 minutes. Random fields (50× magnification) were selected, and cells were counted and photographed.

Wound healing assay

Cells were seeded at a density of 5×10^5 into 6-well tissue culture plates and labelled on the exterior of the culture dishes. After 24 hours of growth, when they reached approximately 70-80% confluency, the monolayer was scraped at the centre of the well using a fresh 200 μl pipette tip perpendicular to the plate and scratched in a straight line in one direction. The cells were then washed twice with phosphate-buffered saline to remove non-adherent cells, and serum-free RPMI 1640 medium was added to the plates. The cells were incubated for 48 hours, at which point a photo of the monolayer at the scratch site was taken under a microscope (50× magnification).

Statistical analysis

Statistical analyses were performed using the SPSS statistical software package (version 25.0), and all data are presented as the means ± SEM (standard deviation) unless otherwise specified. Comparisons were performed between two groups by Student's t-test and between multiple groups by one-way ANOVA. Nonparametric tests were performed to com-

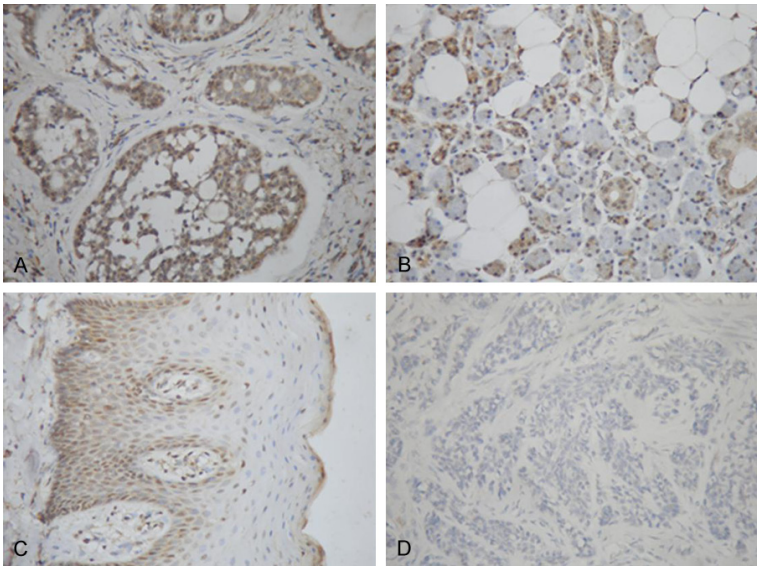


Figure 1. IHC results show that GSDMD expression was significantly higher in ACC than in ANC tissues. A. GSDMD is highly expressed in ACC (100× magnification). B. GSDMD is weakly expressed in ANC tissues (100× magnification). C. Positive control of GSDMD expression in the nuclei of oral mucosal squamous epithelial cells (++) (100× magnification). D. Negative control of GSDMD expression in normal salivary tissues (-) (100× magnification).

pare GSDMD expression between two independent groups. The Mann-Whitney U test and chi-square test were conducted to compare protein expression and clinicopathologic characteristics. The Mann-Whitney U test was performed for the comparison of GSDMD expression between the tumour and ANC tissues, whereas the chi-square test was used to assess differences in protein expression among three types of tissue before the Mann-Whitney U test could be applied for further comparison. All of the statistical tests were 2-sided, and $P<0.05$ was considered significant.

Results

GSDMD is more highly expressed in ACC than in ANC tissues

We first investigated GSDMD expression in patient samples. The results showed that GSDMD expression in ACC was significantly higher than that in either ANC tissues or PA tissues (**Figure 1**). At the same time, the expression of GSDMD in the PA tissues was higher than that in ANC tissues (**Table 1**). Oral mucosal squamous epithelium was used as a positive control for GSDMD expression, and normal salivary tissues were used as the negative control. This result implied that the higher the degree of malignancy is, the higher the expression of GSDMD.

High expression levels of GSDMD are associated with invasion in ACC cell lines

Whether GSDMD affects ACC function remains unknown. To address this issue, GSDMD-overexpressing stable cell lines were constructed in both ACC-LM and ACC-83 cells by lentiviral transfection, and RT-PCR verified that GSDMD-overexpressing cells were successfully established (**Figure 2**). We found that GSDMD was associated with ACC metastatic ability. Both transwell and wound healing assays showed that the GSDMD-overexpressing cell lines displayed significantly stronger metastatic ability than the respective control cell lines. In the scratch assays, smaller gaps in the wound of both GSDMD-ov cell lines were observed after 24 hours (**Figure 3A**). Transwell assays showed that more of the GSDMD-ov cells than control cells passed though the membrane after 48 hours (**Figure 3B**). These results indicate that GSDMD-overexpressing cells have higher invasion activity than control cells.

Downregulation of high levels of GSDMD rescues the change in invasion in adenoid cystic carcinoma

To confirm whether the observed changes are caused by GSDMD, we downregulated the levels of GSDMD expression in the GSDMD-ov cell lines as a rescue. Two siRNAs (siRNA-1 and siRNA-2) targeting GSDMD were transfected, and GSDMD expression was detected by RT-PCR (**Figure 4**). Next, we repeated the transwell and wound healing experiments as described above. The GSDMD-ov cell lines exhibited higher migration and invasion than the respective control cells, but when GSDMD was knocked down in the GSDMD-ov cells, their metastatic abilities were weakened. Compared with GSDMD-ov cell lines, the siRNA-treated GSDMD-ov showed reduced migration and invasion abilities closer to those of the control

Table 1. Results of immunohistochemistry

	Expression				x^2 value	*P*
	Negative	Weakly positive	Positive	Strong positive		
adjacent non-cancerous tissues	9	24	0	0	22.285	P<0.001
pleomorphic adenoma	6	16	7	0		
ACC	0	21	10	2		

The x^2 value is calculated by Pearson's chi-squared test.

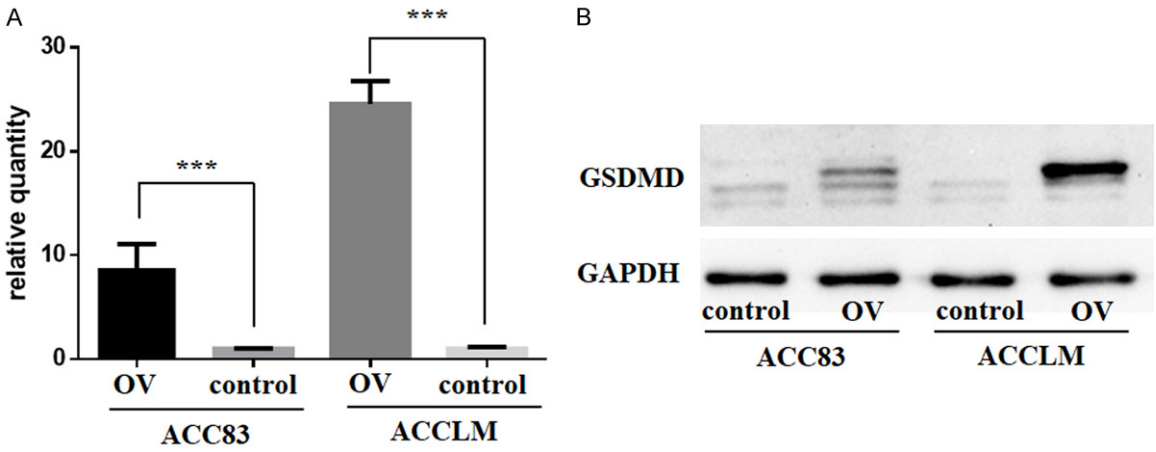
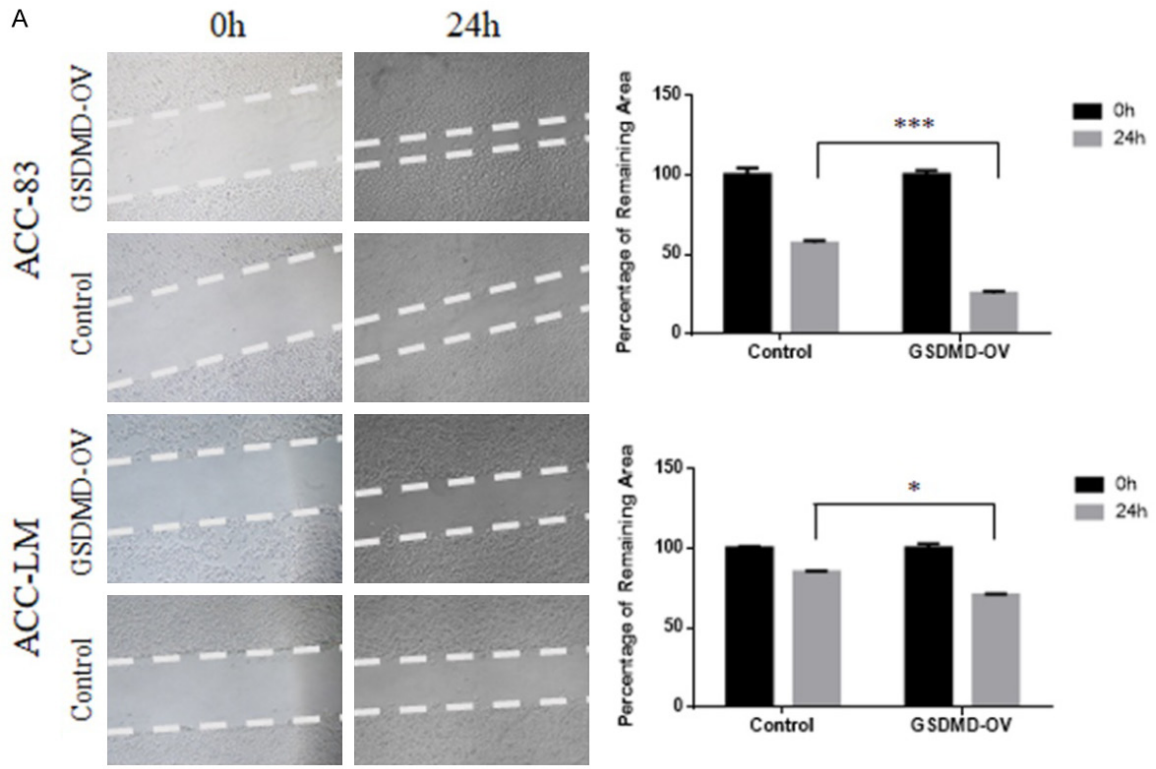


Figure 2. Verification of stable cell lines by western blot and qPCR. A. qPCR of the stable cell lines. The relative expression level of GSDMD in the stable cell line is dozens of times that in the control. B. Western blot of the stable cell lines. The relative expression of GSDMD in the stable cell lines were significantly higher than that in the control cells (Quantitative data are expressed as the means and standard deviation (S.D.) from three replicate experiments. *P<0.05; **P<0.01; ***P<0.001 compared with the control).



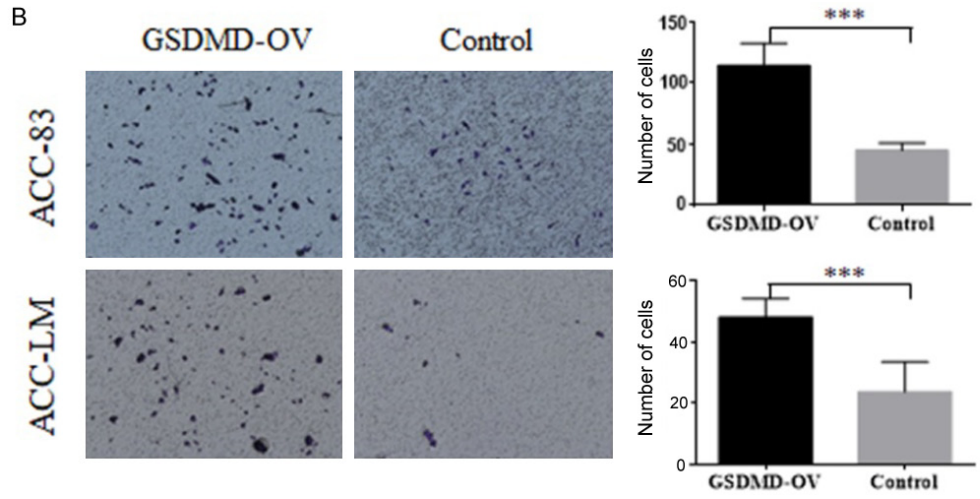


Figure 3. GSDMD overexpression enhanced the metastatic abilities of two ACC cell lines. A. Wound healing results for the GSDMD-ov ACC-83 and ACC-LM cell lines. The GSDMD-ov cells recovered faster than the control cells (50× magnification). B. Cell migration assays for the GSDMD-ov ACC-83 & ACC-LM cell lines. The number of cells that passed through the membrane in the GSDMD-ov group was greater than that in the control group (50× magnification) (Quantitative data are expressed as the means and S.D. from three replicate experiments. *P<0.05; **P<0.01; ***P<0.001 compared with the control).

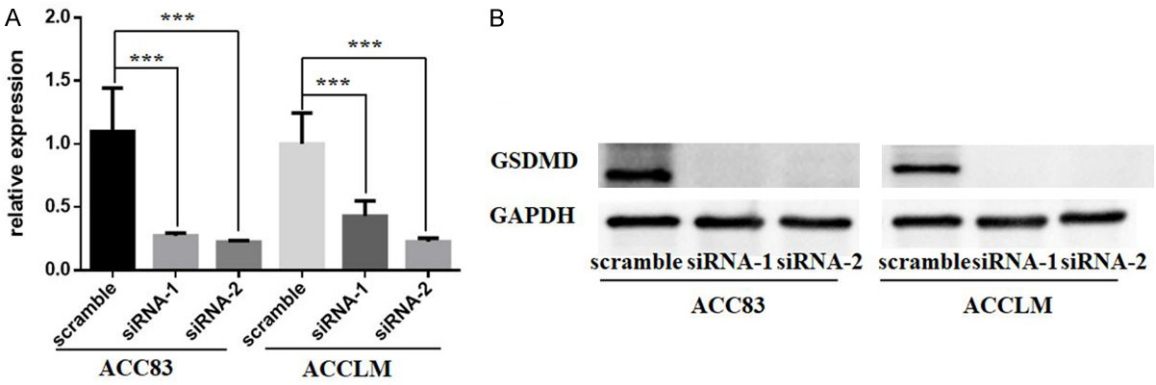


Figure 4. Verification of siRNA-mediated knockdown of GSDMD with siRNA-1 and siRNA-2 in stable GSDMD-ov cell lines by western blot and qPCR. A. The relative expression of GSDMD in the stable cell lines was significantly higher than that in the corresponding stable cell lines transfected with siRNA (both siRNA-1 and siRNA-2). B. Western blot analysis of GSDMD expression in stable GSDMD-ov cell lines transfected with GSDMD-targeted siRNA (both siRNA-1 and siRNA-2) or scramble siRNA. The relative expression of GSDMD in the stable cell lines transfected with GSDMD siRNA (both siRNA-1 and siRNA-2) was significantly lower than that in cells transfected with scramble (Quantitative data are expressed as the mean and S.D. by three replicate experiments. *P<0.05; **P<0.01; ***P<0.001 compared with the control).

cells (**Figure 5**). Hence, these data suggest that GSDMD expression is associated with ACC migration and invasion.

Discussion

ACC accounts for approximately 1% of all malignant tumours of the head and neck and 10% of all salivary gland tumours [24]. Over the years, surgery-based comprehensive approaches have resulted in some progress in the treatment of ACC [25]. However, the treatment effect is still not satisfactory, as relapse and metastasis still occur occasionally [26].

With advancements in research, GSDMD was found to have other functions. As we showed in the results, GSCMD was more highly expressed in ACC than in ANC tissue, which suggests that the degree of GSDMD expression is related to

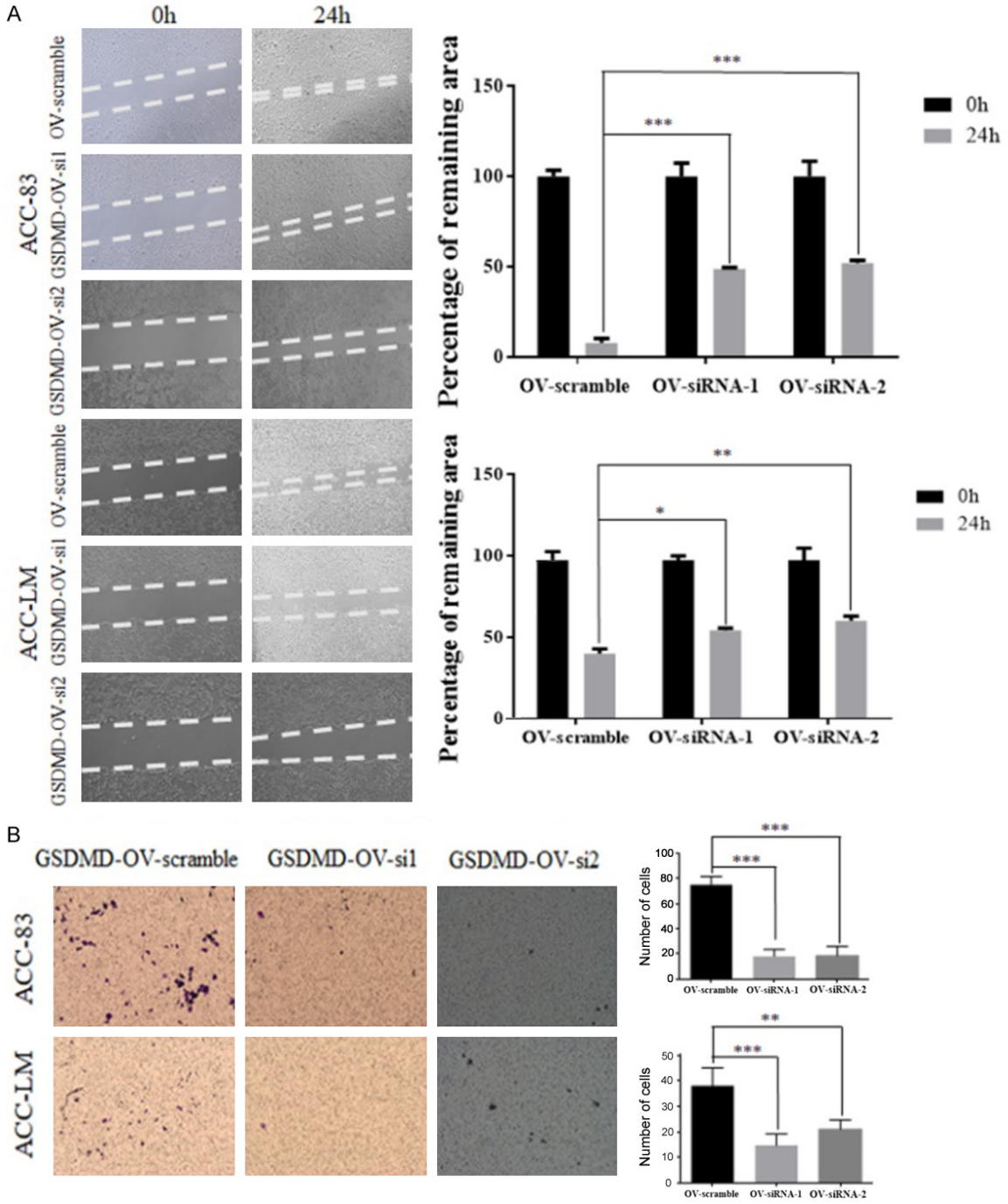


Figure 5. siRNA (both siRNA-1 and siRNA-2) ameliorated the enhanced metastasis abilities of ACC cells with GSDMD overexpression. A. Wound healing results for the GSDMD-ov ACC-83 and ACC-LM cell lines transfected with siRNA (both siRNA-1 and siRNA-2). The GSDMD-ov-siRNA-1 and GSDMD-ov-siRNA-2 groups showed slower closure of the wound than did the GSDMD-ov group (50× magnification). B. Cell migration assays for the GSDMD-ov ACC-83 and ACC-LM cell lines transfected with siRNA (both siRNA-1 and siRNA-2). The number of cells that passed through the membrane in the GSDMD-ov group was greater than that in the GSDMD-ov-siRNA groups (50× magnification) (Quantitative data are expressed as the means and S.D. from three replicate experiments. *P<0.05; **P<0.01; ***P<0.001 compared with the control).

the malignant behaviour of ACC. GSDMD, a protein commonly associated with inflammation, may have an effect on tumours independent of its inflammatory properties.

We upregulated GSDMD expression to study its relationship with invasion and to monitor changes in cell activity. We observed a significant change in the invasive ability of cells with GSDMD overexpression, and we confirmed that this change was caused by GSDMD expression by knocking down GSDMD. The reasons for the increased invasion and whether this activity occurs in vivo need to be further studied, as our results suggest an interesting phenomenon. Although it is known as an important pyroptosis effector protein, GSDMD may have other functions, such as promoting cell invasion. Research in this area is not yet complete, and elucidating the phenomena we observed and their underlying mechanisms may be able to fill the gaps in GSDMD functional research and provide ideas for subsequent exploration of the mechanism(s) involved.

Previously, most studies focused on the effects of pyroptosis and the consequently released cytokines on the malignant behaviour of tumours [15, 27]. In gastric cancer, interleukin 18 is thought to be closely related to distant metastasis of tumour cells [28]. In breast cancer, studies have suggested that high expression of interleukin 18 can promote breast cancer invasion and metastasis [29]. At the same time, other reports have shown that in tongue squamous cell carcinoma, interleukin 18 can promote invasion and metastasis [30]. After the occurrence of pyroptosis, high levels of interleukin 1β and interleukin 18 are released, which may contribute to the promotion of tumour invasion [31]. However, in our study, when GSDMD was highly expressed, the invasive ability of ACC was enhanced. At the same time, no significant cell death occurred, so we speculate that GSDMD-induced cleavage was reduced or absent; therefore, pyroptosis had not yet occurred, and interleukin 1β and interleukin 18 were not released. These results indicate that GSDMD promotes the invasive ability of ACC independent of pyroptosis-mediated release of interleukin 1β and interleukin 18 [32]. Our findings also suggest that there is a more direct path involved, indicating that GSDMD plays a role in promoting ACC invasion before pyroptosis. Identifying this pathway will be the focus of future studies.

Previous work reported that, in gastric adenocarcinoma, GSDMD-induced gastric adenocarcinoma cells upregulated tumour invasion through the interleukin 1β-p38-MMP-2 (matrix metalloproteinase-2) and MMP-9 axes [28]. Our study differs from previous reports in that although GSDMD was highly expressed, no cleavage occurred, no pyroptosis occurred to release interleukin 1β, and no activation of the interleukin 1β-p38-MMP-2/MMP-9 axes was observed [33, 34].

However, there are no reports on whether GSDMD can affect tumour malignancy. We found that ACC migration and invasion were strengthened, which indicates that in addition to the indirect pathway of the interleukin 1β activation axis by the cochlea, GSDMD also has a more direct signalling pathway to enhance tumour migration and invasion. This approach is novel and should be further studied.

Conclusion

GSDMD was highly expressed in ACC tissues compared to ANC tissues, and high GSDMD expression promoted invasion of ACC cells. Knockdown of high levels of GSDMD rescued the invasive changes in ACC. GSDMD may have other functions in addition to pyroptosis, such as promoting the invasion ability of ACC. High GSDMD expression may suggest poor prognosis of ACC.

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Disclosure of conflict of interest

None.

Abbreviations

SACC, salivary adenoid cystic carcinoma; ACC, adenoid cystic carcinoma; GSDMD, gasdermin D; MMP, matrix metalloproteinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; -ov, overexpressed; ANC, adjacent non-cancerous; PA, pleomorphic adenomas.

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