Zhang *et al.*
European Journal of Medical Research (2025) 30:413
https://doi.org/10.1186/s40001-025-02666-5

European Journal
of Medical Research

RESEARCH

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Matrix stiffness enhances viability, migration, invasion and invadopodia formation of oral cancer cells via PI3K/AKT pathway in vitro

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Abstract

Background Oral cancer (OC) is one of the major types of cancer and the most common cause of cancer-related mortality in Asia. In recent years, matrix stiffness in the tumor microenvironment has been found to play an important role in regulating tumor cell behavior. However, the regulatory mechanisms associated with matrix stiffness in OC cells remain unclear.

Methods In this study, polyacrylamide gels with different stiffness were prepared to simulate low versus high matrix stiffness environments in tumor tissues by adjusting the acrylamide and cross-linker concentrations. Subsequently, the effects of different stiffness on OC cell survival, migration, invasion and invadopodia formation were explored based on cell counting kit-8 (CCK-8), Transwell and confocal microscopy. Meanwhile, the levels of markers relevant to phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), apoptosis (BAX and BCL2) as well as metastasis (Cadherin-1, CDH1; Cadherin-2, and CDH2) were calculated via western blotting and real-time quantitative PCR.

Results According to the results, high matrix stiffness was seen to contribute to the increased number of migrated and invaded cells as well as the enhanced viability of OC cells, along with the aggravated invadopodia formation and the up-regulation in CDH2 and BCL2 levels yet the down-regulation in CDH1 and BAX levels. Elevated PI3K/AKT phosphorylation levels were also seen in high matrix stiffness-mediated OC cells, and the intervention using LY294002 could visibly overturned the effects of high matrix stiffness on the cell migration, invasion and invadopodia formation of OC cells.

Conclusions This study reveals that matrix stiffness may enhance the invasiveness and anti-apoptotic ability of OC cells by activating the PI3K/AKT pathway, which provides a new idea for exploring the microenvironmental regulation of tumor mechanics and targeted intervention strategies.

Keywords Matrix stiffness, Oral cancer, Metastasis, Invadopodia, PI3K/AKT pathway

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Introduction

Oral cancer (OC), as a major type of malignancy, is primarily represented by oral squamous cell carcinoma, which accounts for approximately 378,000 new cases and 178,000 deaths worldwide each year [1]. OC is currently creating an alarming situation characterized by the growth of cancerous tissue within the oral cavity and it is a serious matter of concern around the world due to its relatively high incidence and mortality [2–4]. At present, the available treatment methods for OC in clinical practice, including the surgery and radio/chemotherapy, have been applied; however, these therapies have been also reported to have some limitations and hence may not be suitable for the treatment of OC [5]. Metastasis of OC is a complicated process involving the detachment of cells from tumor tissues as well as the modulation on cell motility and invasion, proliferation and evasion through the lymphatic system [6]. This process is primarily driven by mechanisms, such as epithelial–mesenchymal transition (EMT), extracellular matrix degradation, tumor microenvironment (TME) remodeling, and activation of signaling pathways including phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) [7–10].With the aim to improve the survival rate of patients, the prevention of metastasis has become one of the most important issues in clinical practice, which has urged a comprehensive understanding on the relevant mechanisms so as to solve the issue of metastasis [11].

Recent evidences have further supported that matrix stiffness, or increasing tissue rigidity, is implicated in the steps initiating the metastasis including nonrandom survival of a few cell subpopulations, the formation and invasion of invadopodia and the establishment of a TME [12–14]. Notably, increased matrix stiffness leads to the promotion on invasion and metastasis of cancer cells through activating EMT and the relevant pathways [15]. Specifically, both in vivo and in vitro experimental alternations within matrix stiffness can evidently repress tumor growth and invasive metastasis, hinting that the possibility of targeting of matrix stiffness as a therapeutic strategy for cancer in clinical practice [16]. The effects of matrix stiffness on promoting the stemness of glioma cells have been already documented, for instance, [17]. Meanwhile, matrix stiffness triggers EMT via Piezo1-regulated calcium flux in prostate cancer cells [18]. Furthermore, matrix stiffness can epigenetically modulate the oncogenic activation of the Yes-associated protein (YAP) in gastric cancer [19]. Considering the association between the matrix stiffness and the increased risk of cancer development and progression, pharmacological intervention on matrix stiffness may become an option for the prevention and treatment of cancers [7]. Stiff polyacrylamide gel can be applied to reflect the stiffness of tumorous tissues which promote the mobility and invasiveness of cells (where PI3K/AKT pathway takes part) [20, 21]. Herein, our current study begins to explore the potential impact of matrix stiffness on the malignant behaviors of OC cells and investigate the involvement of PI3K/AKT pathway. In this study, we constructed polyacrylamide gel microenvironments with different stiffnesses to simulate the mechanical properties of tumor tissues, systematically evaluated the effects of high matrix stiffness on OC cell activity, migration, invasion, and invadopodia formation, and verified the key role of the PI3K/AKT signaling pathway in the process by combining with the intervention of the inhibitor LY294002. Overall, this study reveals the mechanism by which high matrix stiffness regulates the malignant behavior of OC through the PI3K/AKT pathway, which has important theoretical value and potential clinical guidance.

Materials and methods

Reagents and KITS

Eagle's minimal essential medium (M475846), penicillin–streptomycin (P485293), sodium hydroxide (NaOH, S431793), (3-Aminopropyl) triethoxysilane (APTS, A107148), glutaraldehyde solution (G598815), acrylamide (A108465), bis-acrylamide (M104027), dichlorodimethylsilane (D104810), ammonium persulfate (A112451), tetramethylethylenediamine (TEMED, T140800), sulfo-SANPAH (S276259), collagen I (C390611), nitric acid (N707052), gelatin (G274269), sodium borohydride (S108355), ethanol (E111991), 4% paraformaldehyde (P395744), 0.1% Triton X-100 (T109026), methanol (M116115) and crystal violet (C408642) were all ordered from Aladdin (Shanghai, China). Fetal bovine serum (FBS, S9030), cell counting kit-8 (CCK-8) assay kit (CA1210), Matrigel basement membrane matrix (M8370), poly-L-lysine (P2100), bovine serum albumin (IA0910), 4',6-diamidino-2-phenylindole (DAPI, C0065), AKT inhibitor LY294002 (IL0270), DMSO (D8370), phosphate buffered saline (PBS, P1020) and TBST (T1085) were purchased from Solarbio Lifesciences (Beijing, China). Alexa Flour™ 647 phalloidin (A22287), Total RNA extractor TriZol (15596026), cDNA synthesis kit (K1621), SYBR Green qPCR Mix (4309155), RIPA buffer (89900), BCA protein assay kit (23225), SDS–PAGE separation gel (89888), PVDF membranes (88585), and ECL western blotting substrate (32209) were bought from ThermoScientific (Waltham, MA).

Cell culture and intervention

Human OC cell line HSC-3 (JCRB0623) were available from JCRB Cell Bank (Osaka, Japan) and cultured in the culture medium Eagle's minimal essential medium with the supplementation of 10% FBS and 1%

penicillin–streptomycin at 37 ℃ with 5% CO_2. HSC-3 cell line has been authenticated by STR analysis and tested negative contamination of mycoplasma.

For the intervention of AKT inhibitor, OC cells were additionally treated with 20 μM LY294002 pre-dissolved in DMSO for 24 h [22]. Those treated with equivalent volume of PBS were applied as the negative control.

Polyacrylamide gel preparation

The polyacrylamide gel for this study was prepared based on an existing study and the brief procedures are listed in Fig. 1A [23]. The composition of polyacrylamide gels with low or high stiffness is shown in Fig. 1B for reference. In detail, 50 μL NaOH (0.1 M) was applied to cover the 25-mm circular coverslips. Following 5 min, these coverslips were activated using 50 μL APTS, carefully rinsed and incubated with 0.5% glutaraldehyde in PBS. Subsequently, these coverslips were washed and air-dried. For the polymerization, acrylamide, bis-acrylamide and distilled water at the desired concentrations were mixed together for 15 min and added onto the chloro-silanated glass slides treated with dichlorodimethylsilane. Ammonium persulfate and TEMED were next serially added for gel polymerization for 30 min. Sulfo-SANPAH (0.2 mg/mL) was applied to functionalize the gels under an ultraviolet light at 365 nm for 30 min, followed by the final addition of collagen I for 6 h.

For our current study, HSC-3 cells were further cultured on PA gels with either high or low stiffness for 24 h, aiming to simulate different mechanical microenvironments of the tumor matrix [24]. This setup was used to evaluate the effects of matrix stiffness on cell viability, migration, invasion, and invadopodia formation under controlled in vitro conditions.

Transwell migration/invasion assay

Here, transwells pre-coated with Matrigel can mimic the extracellular matrix barrier and thus be used to assess

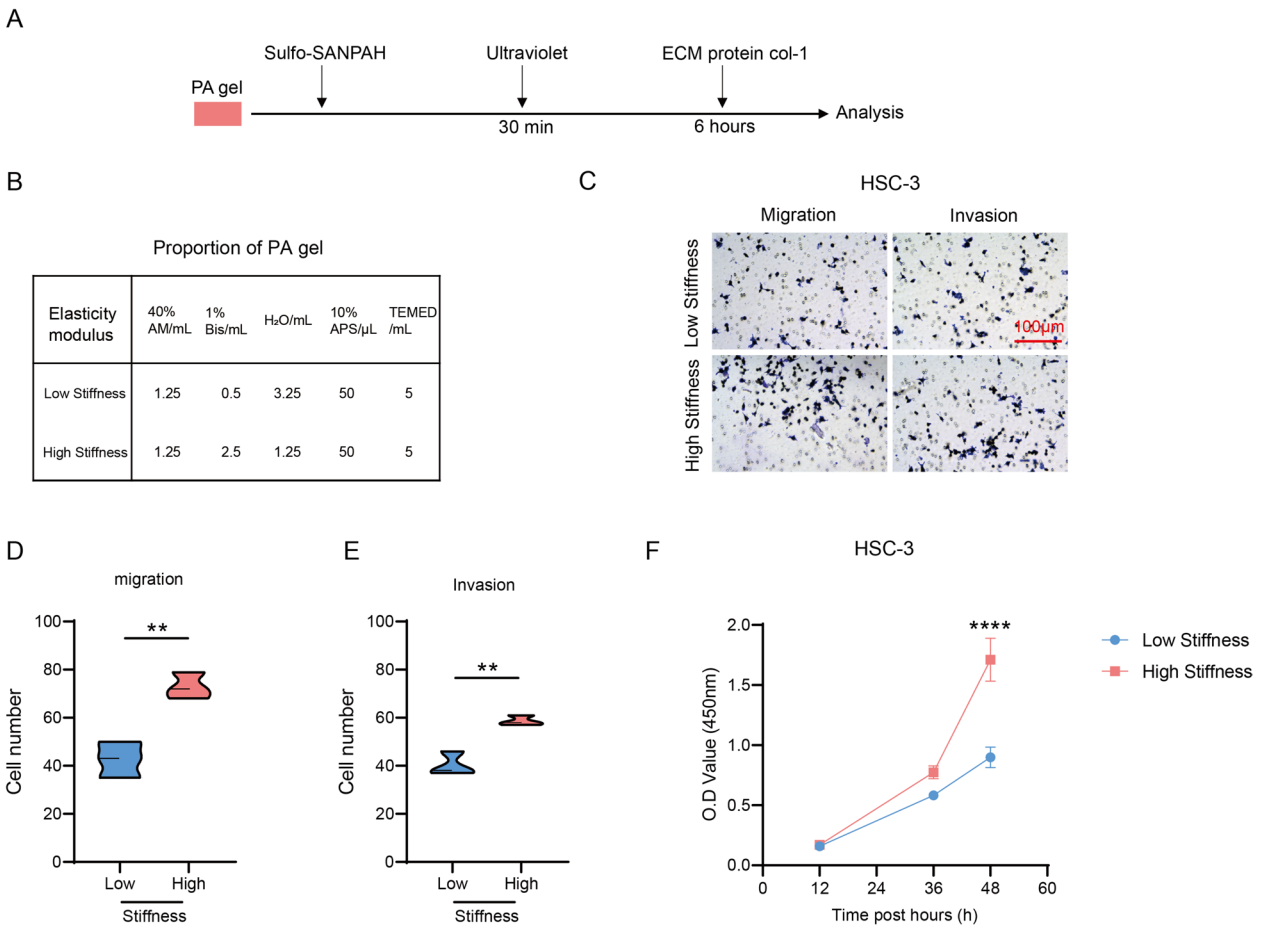

Fig. 1 High matrix stiffness enhanced the migration and invasion of oral cancer cells in vitro. **A** Indicated processes showing the preparation of polyacrylamide gels. **B** Proportion of polyacrylamide gels at different stiffness degrees. **C**–**E** Transwell migration/invasion assay determining the migration and invasion of oral cancer cells at 48 h. **F** CCK-8 assay demonstrating the viability of oral cancer cells at different timepoints. The statistical analyses were implemented with Student's *t* test or two-way analysis of variance (ANOVA). $^{**}p < 0.01$; $^{****}p < 0.0001$

the invasive ability of tumor cells to cross the matrix. The transwell invasion assay was implemented using the 24-well Transwell plates (pore: 8 μm, 3422, Corning, Inc., Corning, NY) coated with the Matrigel matrix, while the transwell migration assay was carried out using the same plates without the Matrigel matrix. OC cells (4×10^4) were seeded in the upper chamber with the non-serum culture media (200 μL), and 600 μL complete medium containing 10% FBS was added to the lower compartment as the chemoattractant. After 48 h, the non-migrating/non-invading cells in the upper chamber were carefully removed using a cotton swab, and the lower chamber was fixed in methanol and stained with crystal violet staining solution. An inverted optical microscope (Eclipse Ti2-A, Nikon Instruments Inc., Tokyo, Japan) was applied to observe three randomly picked area, and the number of migrating/invading cells was accordingly calculated [25, 26].

Cell viability assay

Measurement of cell viability in different groups was conducted with the commercial CCK-8 assay kit. OC cells at a density of 2×10^3 cells per well were distributed in 96-well plates at 37 ℃ with 5% CO_2 for 0, 12, 24, 36 and 48 h. Hereafter, the supernatant was discarded and 10 μL CCK-8 solution was added to each well for 4-h incubation. A microplate reader (iMark, Bio-Rad, Hercules, CA) was applied to read the absorbance at 450 nm [27].

Invadopodia formation assay

Invadopodia formation assay was implemented based on a published study [28]. In detail, the coverslips (12 mm) were pre-treated with 20% nitric acid, washed and incubated with poly-L-lysine (50 μg/mL). Subsequently, 0.5% glutaraldehyde was added to the coverslips, which were inverted onto gelatin at a droplet size of 30 μL for 10 min. Then, sodium borohydride (5 mg/mL) and 70% ethanol were applied to treat and sterilize the coverslips, which were incubated in the pre-warmed medium in a 12-well type culture plate for 1 h.

OC cells in a total number of 6×10^4 were seeded on each coverslip and cultured for 24 h. Hereafter, these cells were fixed in 4% fixative paraformaldehyde and permeabilized using 0.1% Triton X-100. 5% bovine serum albumin was used to block the nonspecific binding for 2 h and cells were treated with Alexa Flour™ 647 phalloidin for 30 min and rinsed in PBS. The coverslips were dyed with DAPI and visualized under a confocal microscope (AX, Nikon Instruments Inc., Japan)

Western blotting assay

Total cells were detached and lysed in RIPA buffer and the protein concentrations were hereafter determined via BCA method. Then cell lysates were resolved on prepared separation gels and transferred to the PVDF membranes, followed by the western blotting assay using the primary antibodies [29] (1/1000 dilution, Cell Signaling Technology, Danvers, MA) against phos-PI3K (#4228), PI3K (#4292), phos-AKT (#9271), AKT (#4691), BAX (#5023), BCL2 (#4223), Cadherin-1 (CDH1, #3195), Cadherin-2 (CDH2, #13116) and housekeeping control GAPDH (#5174). Goat anti-rabbit secondary antibody (conjugate: horseradish peroxide, #7074, Cell Signaling Technology) was applied for further culture, and the protein bands were visualized with the ECL western blotting substrate and ChemiDoc imaging system (Bio-Rad). Quantification analyses were carried out in ImageJ (v. 1.42G, National Institute of Health, Bethesda, MD).

Real-time quantitative PCR (RT-qPCR)

Total cellular RNA from the processed OC cells HSC-3 was extracted using 1 μL of the total RNA extractor Tri-Zol, following the manufacturer's instructions. Following the quantification on the concentration in ND-2000 spectrophotometer (ThermoScientific), 1 μg total RNA was applied for the reverse transcription into cDNA using a relevant cDNA synthesis kit. Then the amplified cDNA was subjected to PCR using the SYBR Green qPCR Mix in ABI7500 real-time PCR system (4366605, ThermoScientific) at the following parameters: 95 ℃ for 10 min and 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The primers applied were:

BAX Forward: 5′-GACGAACTGGACAGTAACAT-3′.

BAX Reverse: 5′-CTTCTTCCAGATGGTGAGT-3′.

BCL2 Forward: 5′-GATGACTGAGTACCTGAACC-3′.

BCL2 Reverse: 5′-AGCAGAGTCTTCAGAGACAG-3′.

CDH1 Forward: 5′-GCCTCCTGAAAAGAGAGTGGAAG-3′.

CDH1 Reverse: 5′-TGGCAGTGTCTCTCCAAATCCG-3′.

CDH2 Forward: 5′-CCTCCAGAGTTTACTGCCATGAC-3′.

CDH2 Reverse: 5′-GTAGGATCTCCGCCACTGATTC-3′.

GAPDH Forward: 5′-ATTGACCTCAACTACATGGT-3′.

GAPDH Reverse: 5′-CATACTTCTCATGGTTCACA-3′.

The relative mRNA level was finally calculated as per the $2^{-\Delta\Delta CT}$ method with GAPDH as the housekeeping control [30].

Statistical analyses

All statistical analyses of this study were conducted using GraphPad Prism 7 and the values represent mean ± standard deviation. Graphs comparing two conditions were analyzed with student's *t* test. CCK-8 data were analyzed with two-way ANOVA method. The threshold of statistical significance was defined as $p < 0.05$ (in the figures, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{****}p < 0.0001$).

Results

High matrix stiffness enhanced the viability, migration and invasion of OC cells in vitro

In this study, we first explored the effects of different matrix stiffness on the migration and invasion of OC cells in vitro following the preparation of a collagen-coated polyacrylamide hydrogel system. Relevant data from transwell assay have illustrated that OC cells manifested a pro-migration and pro-invasion phenotype at a high matrix stiffness (Fig. 1C–E, $p < 0.01$). Meanwhile, the results of the CCK-8 assay showed a significant increase in the survival rate of OC cells at high matrix stiffness (Fig. 1F, $p < 0.0001$). These results hence hinted the modulation of different mechanical forces on the viability, migration and invasion of OC cells in vitro.

High matrix stiffness promoted the invadopodia formation in OC cells in vitro

Subsequently, the confocal microscopy was applied to visualize the formation of invadopodia in OC cells with different mechanical forces, where it was seen that increasing matrix stiffness triggered the invadopodia formation so as to contribute to the acquisition of mesenchymal morphology (Fig. 2A, B, $p < 0.01$), indicating the promoting effects of increasing matrix stiffness on the invadopodia formation in OC cells in vitro.

High matrix stiffness activated PI3K/AKT pathway in OC cells in vitro

Then we sought to look into the potential downstream pathway underlying such promoting effects of matrix stiffness on OC cells. In this study, we focused on PI3K/AKT pathway and the relevant proteins expressions were accordingly quantified. Based on western blotting assay, the phosphorylation levels of both PI3K and AKT were visibly increased in OC cells with the increasing matrix stiffness (Fig. 3A–C, $p < 0.001$), which underlined the potential involvement of PI3K/AKT pathway in OC cells with different mechanical forces.

Modulation of high matrix stiffness on metastasis- and apoptosis-related proteins in OC cells in vitro

The expressions of metastasis- and apoptosis-related proteins in OC cells with high or low matrix stiffness were additionally measured with western blotting assay. Based on the quantification, it was clear that high matrix stiffness could evidently promote the levels of pro-survival protein BCL2 and pro-metastasis protein CDH2 but diminish those of pro-apoptosis protein BAX and anti-metastasis protein CDH1 in OC cells (Fig. 4A–E, $p < 0.05$). Hence, increasing matrix stiffness may also affect the proteins related to the apoptosis and migration on OC via up- or down-regulating the relevant mediators.

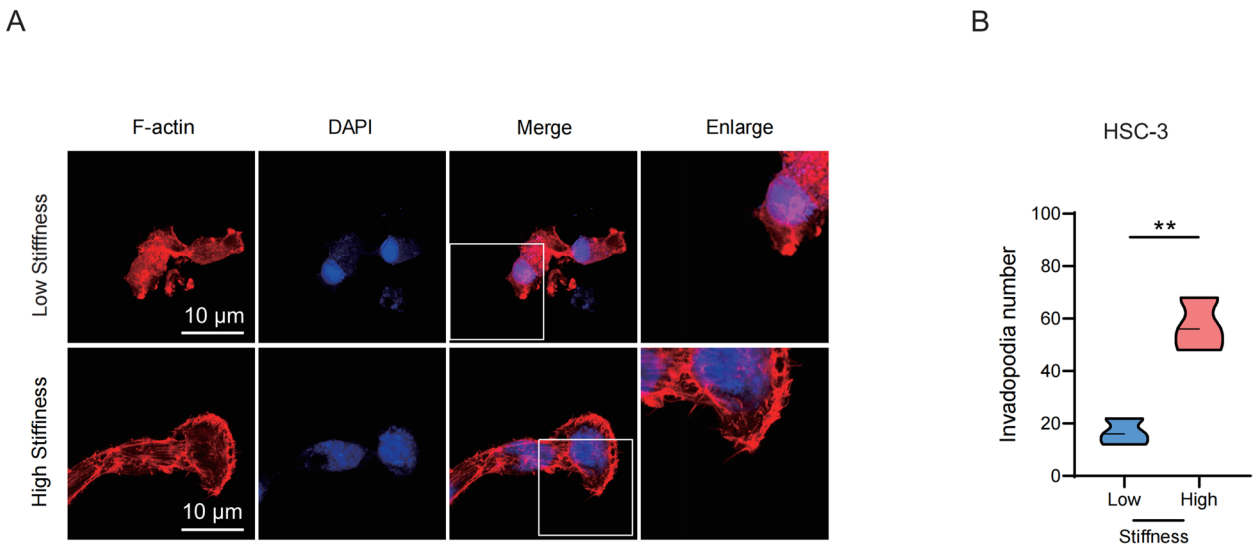

Fig. 2 High matrix stiffness promoted the invadopodia formation in oral cancer cells in vitro. **A** Visualization on invadopodia formation in oral cancer cells with different mechanical forces via confocal microscopy. **B** Quantified number of invadopodia in oral cancer cells based on the results. All statistical analyses were implemented with Student's *t* test. $^{**}p < 0.01$

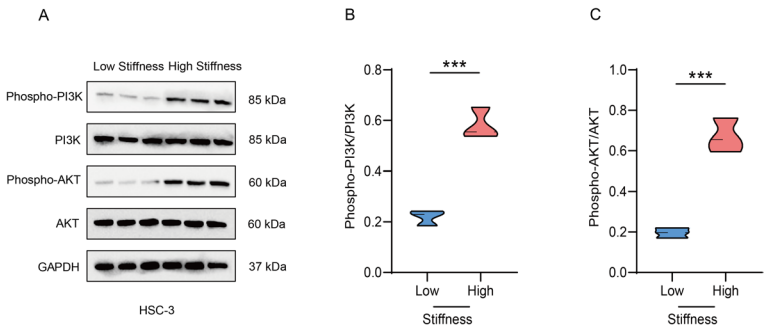

Fig. 3 High matrix stiffness activated PI3K/AKT pathway in oral cancer cells in vitro. **A** Oral cancer cells were exposed to different mechanical forces and the phosphorylation levels of PI3K and AKT were determined based on western blotting assay. **B**, **C** Phosphorylation levels of PI3K (**B**) and AKT (**C**) in accordance with western blotting assay. All statistical analyses were implemented with Student's *t* test. $^{***}p < 0.001$

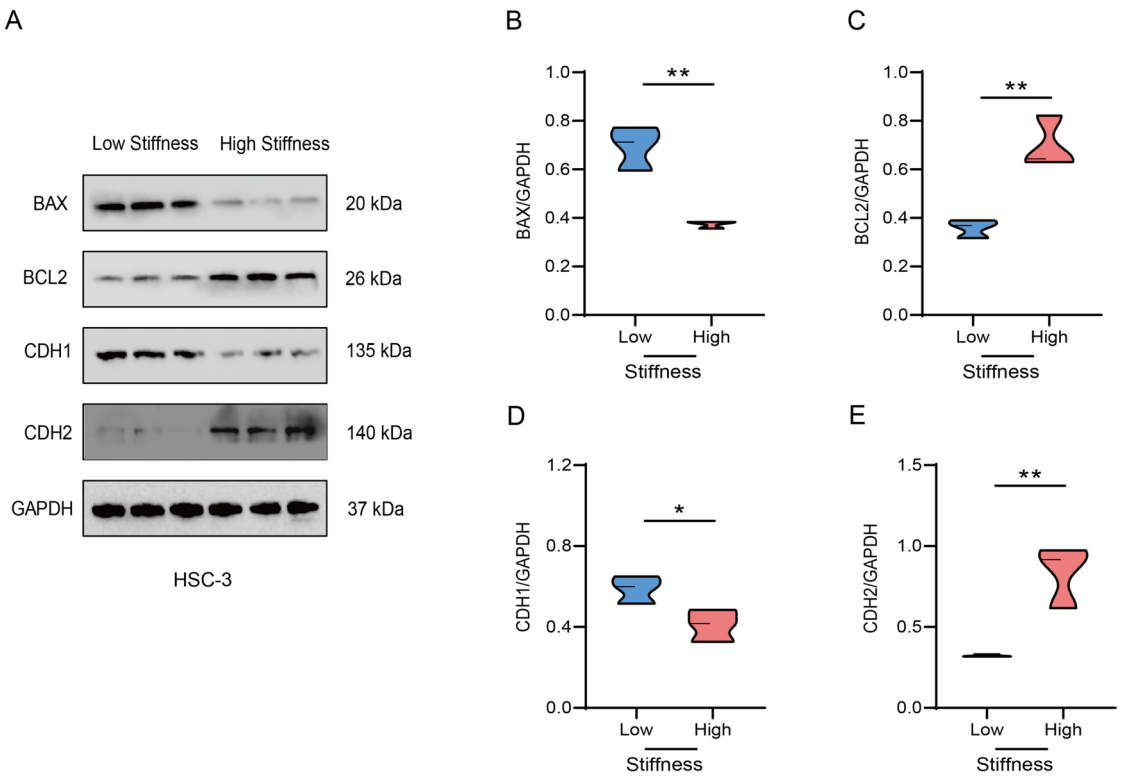

Fig. 4 Modulation of high matrix stiffness on metastasis- and apoptosis-related proteins in oral cancer cells in vitro. **A** Oral cancer cells were exposed to different mechanical forces and the protein levels of metastasis- and apoptosis-related markers in oral cancer cells were accordingly gauged using western blotting assay. **B**–**E** Levels of apoptosis-related markers [BAX (**B**) and BCL2 (**C**)] and metastasis-related markers [CDH1 (**D**) and CDH2 (**E**)] based on western blotting assay. All statistical analyses were implemented with Student's *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$

Inhibition of PI3K/AKT diminished the number of migrated and invaded cells as well as the invadopodia formation in OC cells in vitro

Hereafter, with the purpose of exploring the involvement of PI3K/AKT pathway in OC cells with high mechanical forces, PI3K inhibitor LY294002 was applied to treat OC cells with high mechanical forces. As shown by the Transwell assay, in OC cells with high matrix stiffness, the administration of LY294002 could visibly diminish the number of migrated and invaded cells at the 48 h, as compared to those treated with PBS as the controls (Fig. 5A–C, $p < 0.01$). Confocal microscopy images also showed a decrease in invadopodia formation in OC cells with high matrix stiffness following the intervention of

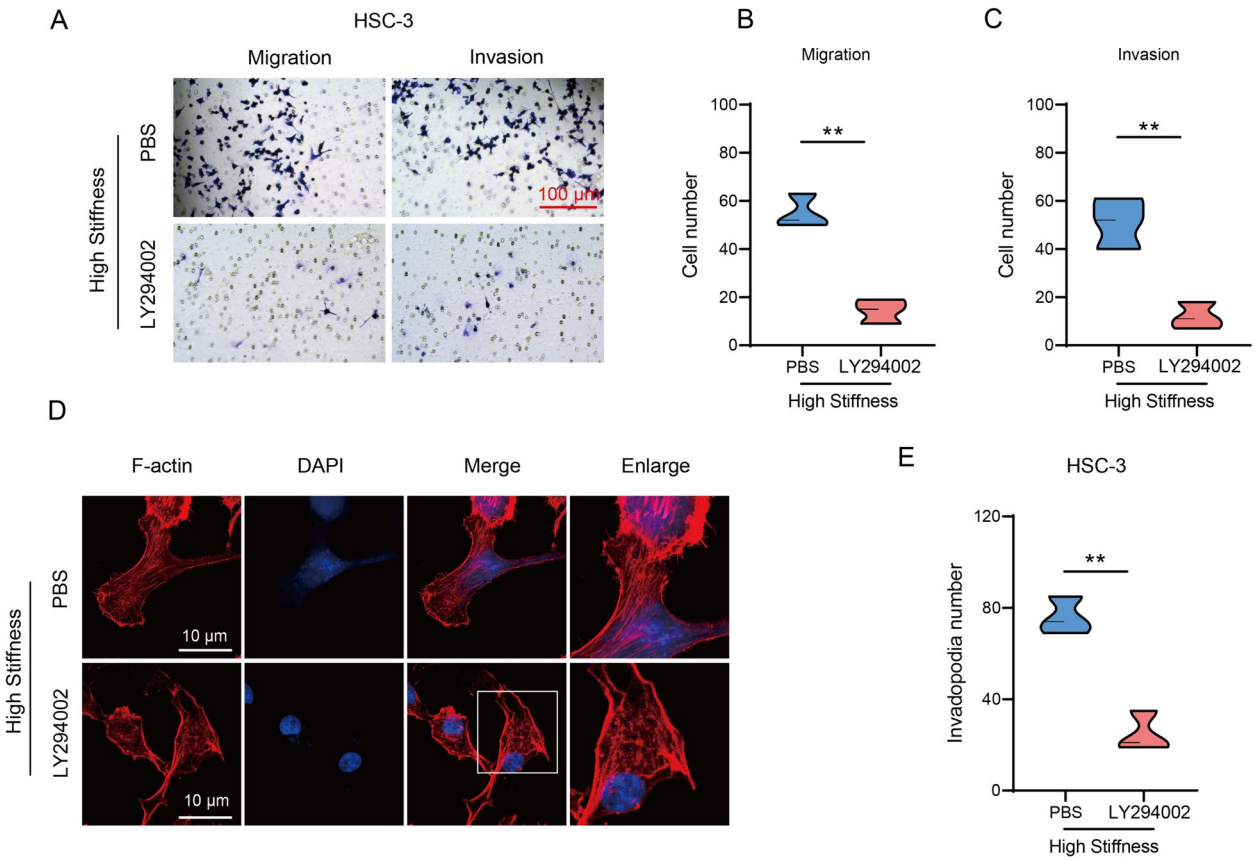

Fig. 5 Inhibition of PI3K/AKT diminished the number of migrated and invaded cells as well as the invadopodia formation in oral cancer cells in vitro. **A**–**C** Transwell migration/invasion assay determining the migration and invasion of oral cancer cells with high mechanical forces and PI3K inhibitor LY294002 at 48 h. **D**, **E** Visualization on invadopodia formation in oral cancer cells with high mechanical forces and PI3K inhibitor LY294002 via confocal microscopy and the quantified number of invadopodia in oral cancer cells. All statistical analyses were implemented with Student's *t* test. $^{**}p < 0.01$

LY294002 in comparison with those treated with PBS (Fig. 5D, E, $p < 0.01$). Collectively, it was suggested that PI3K/AKT may potentially implicate in the promoting effects of matrix stiffness on the migration, invasion and invadopodia formation in OC cells in vitro.

Inhibition of PI3K/AKT regulation of metastasis- and apoptosis-related markers in high matrix stiffness OC cells in vitro

Finally, we used LY294002 to assess the effect of high matrix stiffness on OC cell migration and apoptosis after inhibiting the PI3K/AKT pathway. The results from RT-qPCR have manifested that in OC cells exposed to high matrix stiffness, LY294002 intervention could decrease the expression of BCL2 and CDH2 yet promote those of BAX and CDH1 (Fig. 6A–D, $p < 0.001$). These findings further confirm that the PI3K/AKT pathway is critically involved in mediating the effects of matrix stiffness on the apoptotic resistance and metastatic potential of OC cells.

Discussion

During carcinogenesis, both solid stress and extracellular matrix (ECM) stress can, respectively, interact as the external and internal forces of tumors [31]. Solid stress, by definition, is harbored by the solid phase of tumors, which includes the one exerted by the surrounding normal tissue to repress the expansion of tumors with the expansion of tumors [32]. ECM within the TME is a complicated 3D structure of non-cellular components, which can provide both structural and biochemical support for cells in the vicinity [33–35]. Mechanical signals from the microenvironment have been documented to affect tumors. Stiffness can be converted to mechanical force signals (ECM stiffness, for instance), which can be sensed by cells and then cause the downstream biochemical signaling to take part in invasion and metastasis of malignancies [36]. Here, our current relevant experimental results have demonstrated that increasing ECM stiffness can be a promoter in cell migration, invasion and invadopodia formation in OC cells by PI3K/AKT

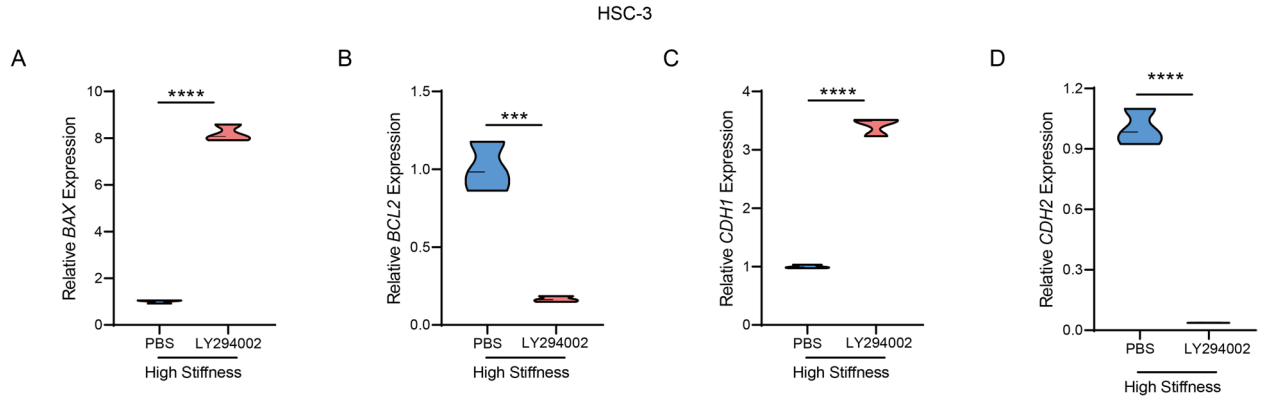

Fig. 6 Metastasis- and apoptosis-related markers in OC cells exposed to high matrix stiffness in vitro. **A**–**D** Levels of apoptosis-related markers [BAX (**A**) and BCL2 (**B**)] and metastasis-related markers [CDH1 (**C**) and CDH2 (**D**)] in oral cancer cells with high mechanical forces and PI3K inhibitor LY294002, quantified via RT-qPCR. All statistical analyses were implemented with Student's *t* test. $^{***}p < 0.001$; $^{****}p < 0.0001$

pathway activation. These results further hinted the role of ECM stiffness and re-proved the involvement of PI3K/AKT pathway in OC.

The ECM has been underscored to interact with tumor and stromal cells so as to enhance the malignant phenotypes of tumor cells, and such relevant mechanisms can be attributed to the mechanical stimuli activating cell membrane receptors and mechanosensors [7]. In other words, cancer cell's malignant phenotypes are the consequences of ECM stiffness [12]. It has been documented that Piezo1 activation could contribute to ECM stiffness-induced angiogenesis in hepatocellular carcinoma [37]. In addition, matrix stiffness puts a trigger on the lipid metabolic crosstalk, thereby mediating bevacizumab resistance in colorectal cancer liver metastasis [38]. Furthermore, matrix stiffness can strengthen the interaction between macrophage and tumors and promote the accumulation of M2-like macrophage in the TME of breast cancer [24]. When it comes to carcinoma of the oral cavity, matrix stiffness conditions EMT mechanically and migratory behavior of oral squamous cell carcinoma and induces an invasive-dormant subpopulation in OC through cGAS–STING axis [39, 40]. In our current study, we additionally expanded the effects of matrix stiffness on the malignant phenotypes of OC cells. In other words, increasing matrix stiffness could promote the migration and invasion of OC cells, concurrent with the increased levels of CDH2 and BCL2 yet the decreased levels of CDH1 and BAX. CDH1 (also known as Epithelial cadherin) is a well-known growth and invasion repressor mainly expressed by epithelial cells, while CDH2 (alternatively known as neuronal cadherin) normally function in non-epithelial tissues which can replace CDH1 during EMT [41–43]. Increasing studies have additionally proven their involvement in cancers including OC, along with their distinct effects [44–46]. BCL2 family, along with their members BCL2 and BAX, for instance, is involved in controlling the apoptosis, with the oncogenic potential of BAX and the anti-apoptotic role of BCL2 of OC [47, 48]. In addition, the invadopodia formation in OC was examined, considering the key role of invadopodia in the maintenance of high migrating and invasive capacities of cells [49]. A conspicuous increased number of invadopodia formed was seen in OC cells with increased matrix stiffness, which was consistent with the trends reported in other cancers, such as hepatocellular carcinoma and nasopharyngeal carcinoma [23, 50].

While examining the potential mechanisms, it has been highlighted that matrix stiffness can contribute to cancer progression through certain transcription factors, such as YAP/transcriptional coactivator with PDZ-binding motif (TAZ), β-catenin and nuclear factor kappa B (NF-κB) [51]. Other studies have revealed that TAGLN could mediate stiffness-regulated progression of ovarian cancer through the RhoA/ROCK pathway and that matrix stiffening can facilitate the invasion of breast cancer via the periostin–integrin mechanotransduction pathway [52, 53]. In our current study, we innovatively put forward that PI3K/AKT pathway may be affected by the different matrix stiffness in OC cells. The crucial role of PI3K/AKT pathway in a variety of cellular processes has been explored, and the aberrant activation in cancers have been reported, thereby contributing to the occurrence and progression of tumors together with its components [54]. PI3 Ks can constitute a lipid kinase family for the generation of phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P(3)), the second messenger, which AKT can interact and modulate, thereby leading to the translocation of AKT to the inner membrane and the subsequent phosphorylation of AKT [55]. The activated AKT

hereafter regulates the functions of substrates implicated in regulating cell survival, growth and cell cycle progression [55]. The targeting of PI3K/AKT pathway as an anti-OC strategy has been already discussed in some papers [10, 56]. In our current study, we witnessed the increased phosphorylation of PI3 K and AKT in OC cells with high matrix stiffness, and further rescue assay has suggested that the inhibition of PI3K/AKT using LY294002 could diminish the number of migrated and invaded cells as well as the invadopodia formation in OC cells, along with the up-regulated expression of BAX and CDH1 and the down-regulated expression of BCL2 and CDH2. Therefore, it could be speculated that matrix stiffness may contribute to the malignant behaviors and invadopodia formation of OC cells through modulating the PI3K/AKT pathway.

However, this study also has certain research limitations. First, all the experiments in this study were done based on the HSC-3 cell line, which can reflect the response of some OC cells to matrix stiffness but fails to comprehensively represent OC cells with different molecular subtypes or different invasive abilities. Therefore, other oral squamous cell carcinoma cell lines should be introduced for parallel validation in the future to enhance the broad applicability and scientific validity of the results. In addition, all the current experiments are in vitro studies, and although they reveal that high stiffness regulates the malignant behavior of cells through the PI3K/AKT pathway, its role in the in vivo tumor microenvironment is not clear. In the future, oral cancer xenograft models with bioscaffolds of different stiffness can be constructed to further evaluate the effects of high stiffness on tumor growth and metastasis in vivo. Finally, although LY294002 is widely used in PI3K/AKT pathway studies, its specificity is still limited and may affect other related signaling axes. We will further incorporate gene disruption (e.g., siRNA or CRISPR–Cas9 technology) to inhibit the expression of PI3K or AKT to further improve the specificity and reliability of the validation.

Conclusion

Overall, our current observation has hinted that the mechanisms, whereby matrix stiffness enhances the malignant behaviors and invadopodia formation of OC cells are related to the activation of PI3K/AKT pathway. Our research revealed that PI3K/AKT inhibition via LY294002 could diminish the matrix stiffness-mediated enhanced migration, invasion and invadopodia formation in OC cells. Nonetheless, it should be noticed that all these results were generated and concluded based on one OC cell line only, and more experimental analyses on other OC cell lines should be incorporated to complete the conclusion we drawn here. In addition, the corresponding molecular mechanisms with regard to invadopodia formation in OC cells are also worth of further exploration.

Abbreviations

OC	Oral cancer
TME	Tumor microenvironment
EMT	Epithelial–mesenchymal transition
YAP	Yes-associated protein
PI3K/AKT	Phosphoinositide 3-kinase/protein kinase B
NaOH	Sodium hydroxide
APTS	(3-Aminopropyl) triethoxysilane
TEMED	Tetramethylethylenediamine
FBS	Fetal bovine serum
CCK-8	Cell counting kit-8
DAPI	4′,6-Diamidino-2-phenylindole
CDH1	Cadherin-1
CDH2	Cadherin-2
RT-qPCR	Real-time quantitative PCR
ECM	Extracellular matrix
TAZ	Transcriptional coactivator with PDZ-binding motif
NF-κB	Nuclear factor kappa B
PI-3,4,5-P(3)	Phosphatidylinositol-3,4,5-trisphosphate

Acknowledgements

None.

Author contributions

All authors contributed to this present work: [ZHZ], [KT] and [CML] concepted and designed the research, [JYR], [XYH] and [JHL] acquired the data. [ZHZ], [JYR] and [CML] analyzed and interpreted data, [CML], [ZHZ], [KT] drafted the manuscript, [ZHZ], [JYR], [XYH] and [CML] revised manuscript for important intellectual content. All authors read and approved the manuscript.

Funding

The authors declare that they have received no funding.

Data availability

The data is available in Figshare https://doi.org/10.6084/m9.figshare.28570940.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 31 March 2025 Accepted: 5 May 2025
Published online: 24 May 2025

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