ORIGINAL ARTICLE

Substrate stiffness regulated migration and invasion ability of adenoid cystic carcinoma cells via RhoA/ROCK pathway

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Abstract

Objectives: Human salivary adenoid cystic carcinoma (SACC) is one of the most common malignant tumours of the salivary gland and has strong migratory and invasive ability, which often lead to poor prognosis and lower survival rate. Tumour tissue tends to stiffen during solid tumour progression. This study aimed to investigate the influence of various substrate stiffness on the migration and invasion of SACC.

Methods: Salivary adenoid cystic carcinoma cell line ACC2 cells were cultured on polydimethylsiloxane substrates (PDMS) with varying stiffness for investigating the effects of substrate stiffness on the activities of MMPs and TIMPs. The underlying mechanism was also explored.

Results: When ACC2 cells were cultured on various stiffness of PDMS, the expressions of matrix metalloproteinases 2 (MMP2), MMP9, MMP14, RhoA, Rac1, Rho-associated protein kinase 1 (ROCK1) and ROCK2 were up-regulated with increasing substrate stiffness, whereas that of tissue inhibitor of matrix metalloproteinase 1 (TIMP1), TIMP2 and TIMP4 were down-regulated with increasing substrate stiffness.

Conclusions: Our results showed that substrate stiffness regulated the activities of MMPs and TIMPs and then modulate migratory and invasive ability of ACC2 cells via RhoA/ROCK pathway. This work indicate that matrix stiffness played an important role in progression of SACC, which not only can help understand the strong invasive ability of SACC, but also suggested that therapeutically targeting matrix stiffness may help reduce migration and invasion of SACC and improve effective therapies.

1 | INTRODUCTION

Salivary adenoid cystic carcinoma (SACC) is one of the most common malignant tumours of the salivary gland, accounting for about 18% of all salivary gland malignancies and about 1% of all head and neck malignant tumours.¹⁻³ Typical clinical and biological characteristics of SACC include perineural invasion, blood vessel invasion and distant metastasis, which often lead to poor prognosis and lower survival rate. SACC has strong ability to migrate and invade. However, the precise mechanisms underlying migration and invasion of SACC are not known.

Clinical studies have shown that the stiffness of the extracellular matrix (ECM) is significantly increased in a variety of cancers.^{4,5} The

stiffness of cancers is higher than normal tissues. For example, the normal breast has the Young's modulus of 0.8-1.2 kpa.⁶ The Young's modulus of breast cancer of different grades and category is from 20 to 200 kpa.⁷ Numerous studies have shown that mechanical changes in the cell microenvironment may be responsible for specific behaviours of cells, such as adhesion, proliferation and migration.⁸ Substrate stiffness has been reported to be powerful regulators of cell migration. MMPs are the main enzymes responsible for ECM remodelling and are classically known to be important drivers of cancer cell migration and invasion.⁹ Changes that affect the expressions of MMPs in cell microenvironment will influence cancer cell migration and invasion. TIMPs are the most important physiological inhibitors of MMP function¹⁰, which inhibit the proteolytic activities of

MMPs by forming 1:1 stoichiometric complexes with active MMPs. However, the relationship between substrate stiffness and expressions of MMPs and TIMPs is few known.

Thus, we investigated in this study, whether substrate stiffness modulates migration and invasion ability of SACC by the expressions of MMPs and TIMPs, using ACC2 cell line and polydimethylsiloxane (PDMS) substrates of varying stiffness. We further want to explore the underlying mechanism.

2 | MATERIAL AND METHODS

2.1 | Preparation of PDMS substrates of different stiffness

Polydimethylsiloxane substrates (Corning, NY, USA) contain two components: oligomeric base and the curing agent Sylgard184. They were mixed together at different ratio (oligomeric base/Sylgard184 = 10:1, 20:1, 30:1 and 40:1) with stirring. A quantity of 1 mL of the thoroughly mixed mixture was then cast on a single-well Petri dish (35 × 10 mm, Corning) or 6-well plates (each well, Corning), and then cross-linked in an oven at 60°C for 24 hours. PDMS substrates were exposed to ultraviolet light for 2 hours to sterilization before cells were cultured onto them.

2.2 | Scanning electron microscope

For observation of cell attachment and morphological changes, ACC2 cells were plated on varying stiffness of PDMS substrates at 1×10^5 cells per well. After fixation in 2.5% glutaraldehyde for 2 hours at 4°C, ACC2 cells were dehydrated in a series of gradient ethanol (30%, 50%, 75%, 85%, 95% and 100%) for 15 minutes at each step. After dried in an exhaust hood, the specimens were mounted on specimen holders, coated with a thin layer of gold and then examined by scanning electron microscope (SEM).

2.3 | Cell culture

The human SACC cell line ACC2 was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Science (Shanghai, China). Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 (Hyclone, Logan, UT, USA) culture media, which was supplemented with 10% foetal bovine serum (FBS, Schaumburg, IL, USA) and 1% penicillin/streptomycin antibiotics (Hyclone, Logan, UT, USA), and were kept in a humidified incubator at 37°C and 5% CO₂ atmosphere.

2.4 | Semi-quantitative polymerase chain reaction (semi-qPCR)

Total RNA were extracted from ACC2 cells at 3 days using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA).¹¹ Isolated RNA was dissolved in RNase-free water and the absorbance at 260 nm was measured with a spectrophotometer to quantify. The reverse

TABLE 1 Sequences of forward and reverse primers of selected genes designed for semi-quantitative PCR

mRNA	Product length (bp)	Primer pairs (5'->3')
<i>GAPDH</i>	101	Forward ACAACTTTGGTATCGTGAAGG Reverse GCCATCACGCCACAGTTTC
<i>MMP2</i>	82	Forward CCCACTGCGGTTTCTCGAAT Reverse CAAAGGGGTATCCATCGCCAT
<i>MMP9</i>	97	Forward TGTACCGCTATGTTTACACTCG Reverse GGCAGGGACAGTTGCTTCT
<i>MMP14</i>	83	Forward GGCTACAGCAATATGGCTACC Reverse GATGGCCGCTGAGAGTGAC
<i>TIMP1</i>	169	Forward AGAGTGTCTGCGGATACTTCC Reverse CCAACAGTGTAGGTCTTGGTG
<i>TIMP2</i>	109	Forward GCTGCGAGTGCAAGATCAC Reverse TGGTGCCCGTTGATGTTCTTC
<i>TIMP4</i>	143	Forward CCACTCGGCACTTGTGATTC Reverse CATCCTTGACTTCTCAAACCT
<i>RhoA</i>	118	Forward GGAAAGCAGGTAGAGTTGGCT Reverse GGCTGTCGATGGAAAAACACAT
<i>Rac1</i>	249	Forward ATGTCCGTGCAAAGTGGTATC Reverse CTCGGATCGCTTCGTCAAACA
<i>ROCK1</i>	93	Forward AACATGCTGCTGGATAAATCTGG Reverse TGTATCACATCGTACCATGCCT
<i>ROCK2</i>	209	Forward TCAGAGGTCTACAGATGAAGGC Reverse CCAGGGGCTATTGGCAAAGG

transcription reaction was performed with the synthesis kit (Mbi, Glen Burnie, MD, USA), with a final volume of 20 µL.

Semi-qPCR was performed with a PCR kit (Mbi) in a 25 µL volume containing 1 µL cDNA sample using a thermocycler (Bio-Rad, Hercules, CA, USA). Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for assessing PCR efficiency. Sets of primers chosen are shown in Table 1. The procedure of PCR consisted of denaturation at 94°C for 30 s, annealing at 55–65°C for 30 s, elongation for 30 s with 28–32 amplification cycles. The products were electrophoresed on 2% agarose gels and visualized by dyeing with GoldView (Heart, Shanghai, China). Quantity One4.6.3 software (Bio-Rad) was used for bands quantification by optical density (OD).

2.5 | Zymography

Zymography was used for detecting the activity of MMP2 and MMP9 in culture media samples (after cultured for 24 hours). Briefly, the protein concentrations were quantified by a BCA kit (Beyotime,

FIGURE 1 Morphology and adhesion of ACC2 cells cultured on polydimethylsiloxane substrates (PDMS) substrates of varying stiffness. Scanning electron micrographs (A-D) and fluorescent images (E) of cytoskeleton (phalloidin staining of F-actin) and vinculin of ACC2 cells plated on PDMS substrates of varying stiffness. The images shown were representative of 3 different experiments ($n = 3$)

FIGURE 2 Gene expressions of matrix metalloproteinases (MMPs) and TIMPs and activity of MMP2/9 in ACC2 cells on polydimethylsiloxane substrates (PDMS) substrates of varying stiffness. The gene expressions of MMP2, MMP9, MMP14, TIMP1, TIMP2 and TIMP4 were detected by semi-qPCR (A). Quantitative analysis of gene expressions was done by the optical densities (OD) method (B-G). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were set as the internal normalized control. Zymography showed different activity of MMP2/9 secreted by ACC2 cells in response to different PDMS substrates (H). Quantitative analysis of the MMP2/9 was done by the OD method (I, J). The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD. *Significant difference with respect to control groups (10:1), * $P < .05$, ** $P < .01$

Nanjing, China).¹² Standard, non-reducing loading buffer containing 62.5 mmol L⁻¹ Tris-HCl, pH6.8, 25% glycerol, 2% sodium dodecyl sulphate (SDS), 0.01% bromophenol blue, no β -mercaptoethanol, was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% SDS-PAGE gel with 0.05% gelatin). Samples were electrophoresed in SDS-PAGE and then SDS was removed from the gel by incubation in 2.5% Triton X-100 three times for 1.5 hours. The gel then was incubated in proteolysis buffer (50 mmol L⁻¹ CaCl₂, 0.5 mol L⁻¹ NaCl, 50 mmol L⁻¹ Tris, pH 7.8) at 37°C for 48 hours. The gel was stained with Coomassie Brilliant Blue for 1 hour and then decoloured in decolourization liquid A, B and C (methanol concentration of 30%, 20% and 10%, respectively; acetic acid concentration of 10%, 10% and 5%, respectively) for 30 minutes, 1 and 2 hours respectively. The OD method was used for quantization of the bands.

2.6 | Western blotting

The whole cell lysis assay (KeyGen Biotech, Nanjing, China) was applied to harvest total proteins.¹³ Loading buffer was added into protein samples. Then, the mixtures were boiled at 100°C for

5 minutes. Proteins were separated in 10% (v/v) SDS-PAGE and then the gel was transferred to a polyvinylidene difluoride membranes at 200 mA for 1 hour. Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) for 1 hour at room temperature (RT) and then incubated with antibodies specific to GAPDH (ab181603), MMP2 (ab37150), MMP9 (ab137867), TIMP1 (ab38978), RhoA (ab187027) and ROCK1 ab134181 (Abcam, Cambridge, UK) overnight at 4°C or 3 hours at RT. Membranes were washed with TBST for 3 times, after which they were incubated with secondary antibody (Beyotime, Shanghai, China) to combine with primary antibody for 1 hour at RT. After washed with TBST for 3 times, blots were developed using a Western Blotting Luminol Reagent Kit (Santa Cruz, CA, USA). Each band was detected by an enhanced chemiluminescence detection system (Bio-Rad).

2.7 | Immunofluorescent staining

After ACC2 cells cultured on the PDMS substrates for 3 days, cells were washed with PBS for 3 times, fixed with 4% cold paraformaldehyde for 20 minutes, perforated using 0.5% Triton X-100 for

FIGURE 3 Protein expressions of matrix metalloproteinases2 (MMP2) and MMP9 in ACC2 cells on polydimethylsiloxane substrates (PDMS) substrates of different stiffness. Immunofluorescence staining showed the expressions of MMP2 (A) in ACC2 cells on PDMS substrates of different stiffness. Western blotting assay analysed the protein expressions of MMP2 and MMP9 in ACC2 cells on different substrate stiffness (B). Quantitative analysis of protein expressions was done by OD method (C,D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were set as the internal normalized control. The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD. *Significant difference with respect to control groups (10:1), * $P < .05$, ** $P < .01$

15 minutes, blocked by 5% normal goat serum for 1 hour at RT and incubated at 4°C overnight with antibodies, MMP2, TIMP1, RhoA and vinculin (ab18058, Abcam, Cambridge, UK). Next day, the cells were rewarmed at RT for 30 minutes, and then incubated with secondary antibody for 1 hour. Then, cells were stained with 50 μ g/mL fluorescent Phalloidin (Sigma-Aldrich, St. Louis, MO, USA) conjugate solution in PBS at RT for 1 hour. Finally, cells nuclei were stained with DAPI for 10 minutes. After each of the

above steps, samples were washed with PBS. Images were captured with confocal laser scanning microscopy (Leica TCSP8, Wetzlar, Germany).

2.8 | Statistical analysis

Each experiment was performed in triplicate and reproduced at least 3 separate times. Mean data of semi-qPCR and western blotting

FIGURE 4 Protein expressions of TIMP1 in ACC2 cells on polydimethylsiloxane substrates (PDMS) substrates of different stiffness. Immunofluorescence staining (A) and western blotting (B) showed the expressions of TIMP1 in ACC2 cells on PDMS substrates of different stiffness. Quantitative analysis of protein expressions was done by optical density (OD) method (C). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were set as the internal normalized control. The results shown were representative of 3 different experiments ($n = 3$). Data were presented as means $\pm$ SD. *Significant difference with respect to control groups (10:1), ** $P < .01$

was first quantified to GAPDH, and then normalized to data of 10:1 groups. A One-way analysis of variance (SPSS 16.0, IBM, Silicon Valley, CA, USA) was used for comparing means of all groups, and the Student-Newman-Keuls testing was used for comparing means of each two groups. If the two-tailed $P < .05$, it can be considered that the data were significantly different.

3 | RESULTS

3.1 | Morphology and adhesion of ACC2 cells cultured on PDMS substrates of varying stiffness

Due to its flexibility, optical transparency, flexibility and low cost, PDMS has been used in many biomaterial studies.¹⁴ PDMS

FIGURE 5 Gene expressions of RhoA/Rho-associated protein kinase (ROCK) pathway related factors in ACC2 cells on substrates of varying stiffness. The gene expressions of RhoA, Rac1, ROCK1 and ROCK2 were detected by semi-qPCR (A). Quantitative analysis of gene expressions was done by OD method (B-E). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were set as the internal normalized control. The results shown were representative of three different experiments ($n = 3$). Data were presented as means $\pm$ SD. *Significant difference with respect to control groups (10:1), * $P < .05$, ** $P < .01$

substrates were used in this study for their wide elastic moduli and good biocompatibility. Our group has tested the stiffness of PDMS substrates formulated for different proportions of bases and curing agents.^{15,16} The stiffness of the PDMS substrates formulated with oligomeric base to Sylgard184 ratios of 10:1, 20:1, 30:1 and 40:1 was approximately 135, 54, 16 and 6 kpa respectively. Our previous studies have observed the morphology and tested the roughness of PDMS substrate of varying stiffness and indicated that no differences in cell behaviour between those cultured on substrates within the roughness.¹⁵ SEM images (Figure 1A-D) showed different morphologies of ACC2 cells cultured on PDMS substrates of varying stiffness. Cells showed more cell invadopodia on the stiffer substrates (10:1 and 20:1), while cells showed fewer cell invadopodia on the softer substrates (30:1 and 40:1). Phalloidin staining for F-actin was used for showing cell cytoskeleton, which indicated that the cell spreading areas were apparently different on substrates of varying stiffness (Figure 1E). ACC2 cells spread widely on stiffer substrates (10:1 and 20:1) and became smaller and rounder on softer substrates (30:1 and 40:1). Vinculin staining showed that cells adhered on the substrates well (Figure 1E). The area of vinculin was larger on the stiffer substrates and smaller on the softer substrates.

3.2 | MMPs and TIMPs expressions in ACC2 cells on PDMS substrates of different stiffness

In order to investigate whether the substrate stiffness could influence the migratory and invasive ability of ACC2 cells, the gene

expressions of MMPs and TIMPs were analysed by semi-qPCR. The 10:1 groups were set as control in all data analyses. The result of semi-qPCR (Figure 2A-G) showed that the expressions of *MMP2*, *MMP9* and *MMP14* decreased, whereas the expressions of *TIMP1*, *TIMP2* and *TIMP4* increased with decreasing substrate stiffness. There was no significant difference in the expressions of *MMP1*, *MMP3*, *MMP7*, *MMP10*, *MMP11*, *MMP12*, *MMP13* and *TIMP3* (the results were not shown).

Matrix metalloproteinases 2 and MMP9 activity in ACC2 cells was detected by zymography (Figure 2H). Quantification of the data (Figure 2I,J) showed that MMP2 and MMP9 secretions declined with decreasing substrate stiffness.

Matrix metalloproteinases 2 and TIMP1 protein levels in ACC2 cells were analysed by immunofluorescent staining (Figure 3A,B) and western blotting (Figures 3C, 4D). The results were consistent with those of gene expression analyses. The protein expressions of MMP2 and MMP9 decreased and that of TIMP1 increased with decreasing substrate stiffness. The results of immunofluorescent staining targeting MMP2 and TIMP1 were consistent with those of western blotting (Figure 4A-C).

3.3 | ACC2 cells sense substrate stiffness and then modulate MMP activity may via RhoA/ROCK pathway

Numerous studies have shown that Rho family plays a significant role in the regulation of cytoskeleton, which is closely related to tumorigenesis, invasion and metastasis.^{17,18} Therefore, for exploring

FIGURE 6 Protein expressions of RhoA and Rho-associated protein kinase (ROCK)1 in ACC2 cells. Immunofluorescence staining showed the expressions of RhoA in ACC2 cells (A). Western blotting assay detected the protein expressions of RhoA and ROCK1 in ACC2 cells cultured on different substrate stiffness (B). Quantitative analysis of protein expressions was done by OD method (C,D). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were set as the internal normalized control. The results shown were representative of 3 different experiments ($n = 3$). Data were presented as means $\pm$ SD. *Significant difference with respect to control groups (10:1), * $P < .05$, ** $P < .01$

the mechanism by which substrate stiffness modulated the migration ability of ACC2 cells, RhoA/ROCK pathway was investigated. The result of semi-qPCR (Figure 5A-E) showed that the expressions of *RhoA*, *Rac1*, *ROCK1* and *ROCK2* were decreased with decreasing substrate stiffness. Meanwhile, the results of immunofluorescent staining of RhoA (Figure 6A) and western blotting of RhoA and ROCK1 (Figure 6B-D) were consistent with those of semi-qPCR.

4 | DISCUSSION

Recently, mechanical stimulus in tumour microenvironment has attracted much attention as potent drivers of migration and invasion.¹⁹ It has been shown that MMPs expression increases in various cancers, which often leads to increased migration potential of cancer cells.²⁰ In this study, the results showed that substrate stiffness,

acting as mechanical stimulus, regulated the expressions of MMPs and TIMPs in ACC2 cells.

Matrix metalloproteinases belong to a family of Zn^{2+} -binding and Ca^{2+} -dependent proteolytic enzymes whose basic function is ECM degradation, a process that is required for cell migration and invasion.²¹ MMPs participate in the degradation of numerous ECM molecules, such as collagens, laminins, fibronectin and gelatin, paving a way for cell migration and invasion. In this study, the expressions of MMP2, MMP9 and MMP14 decreased with the decrease in substrate stiffness, as the same as the activity of MMP2 and MMP9—which act to degrade ECM—while the expressions of TIMP1, TIMP2 and TIMP4—which inhibit the activities of MMPs—were just the opposite. Therefore, MMP: TIMP ratio was higher on stiffer substrates than that on softer substrates, which meant that MMPs had stronger ability to degradation of ECM on stiffer substrates. The results of this study may indicate that ACC2 cells acquired higher ability to migrate and invasive on stiffer substrates than that on softer substrates.

Cell behaviours are controlled by extracellular environment and its biophysical properties in the process of physiological and pathological conditions. Cells connect to ECM and sense micro-environment stimulations via large integrin-based complexes called focal adhesions, which consist of vinculin, talin, paxillin and α -actin.²² Vinculin is a F-actin binding protein localized in focal adhesions, participated in cell attachment, cell rearrangement and cell movement. Vinculin is a key protein of focal adhesions that involves in mechanotransduction and mechanical regulation, acting as a bridge between exterior environment and interior cytoskeleton.^{23,24} Immunostaining images of vinculin and F-actin in this study showed that ACC2 cells indeed sensed the difference of substrate stiffness and had distinctive morphology on substrates of diverse stiffness, from widely spreading to small and round. Stiffer substrates provided enough traction for cell spreading and migration.

Previous studies have showed that focal adhesions transmit signalling by the Rho-family small GTPases to reorganize and contract cytoskeleton. Rho GTPase currently consists of three sub-families, Rho, Rac and Cdc42, of which RhoA, Rac1 and Cdc42 were best studied.^{25,26} RhoA and Rac1 has been reported to be implicated in mechanosensing and they thereby were studied in this work.²⁷ Rho GTPase has been identified as a major regulator in cell morphology and migration through its effects on actin filaments.^{28,29} Rho-associated protein kinase is a downstream effector of the small GTPase Rho and plays a significant role in mediating rearrangements of the actomyosin cytoskeleton.^{30–32} When RhoA bound to guanosine triphosphate (GTP), ROCK1 and ROCK2 are activated and then increase cell contractility through attenuating myosin phosphatase activity and enhancing phosphorylation of myosin light chain 2 (MLC2). ROCK also can promote the phosphorylation of adducin and then promote cell migration.^{33,34} Rac1 regulates the formation of lamellipodia and activation of Rac1 is sufficient to drive cell migration. We found that in this study the expressions of RhoA, Rac1, ROCK1 and ROCK2 were higher on stiffer substrates, which were consistent with the results of

MMPs. Numerous studies have demonstrated that Rho/ROCK signalling pathway can regulate the expressions of MMP2, MMP9 and MMP14 and then enhance cell migration.^{35–38} Consequently, a conclusion can be made that ACC2 cells had stronger ability to migrate on stiffer substrates and RhoA/ROCK signalling pathway was participated in the process of mechanotransduction^{39,40}.

In conclusion, we found in this study that ACC2 cells could sense and then respond to substrate stiffness, which in turn regulated activities of MMPs and TIMPs and then modulate migratory and invasive ability of ACC2 cells. RhoA/ROCK signalling pathway was participated in this process. Our results indicate that matrix stiffness played an important role in progression of SACC, which not only can help understand the strong invasive ability of SACC, but also suggested that therapeutically targeting matrix stiffness may help reduce migration and invasion of SACC and improve effective therapies. Based on the results of this study in vitro, in our future study, we will explore the substrate stiffness on the migration and invasion ability of SACC in vivo.

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