rAj-Tspin, a novel peptide from Apostichopus japonicus, exerts anti-hepatocellular carcinoma effects via the ITGB1/ZYX/FAK/AKT signaling pathway

Ying Che
Dalian Medical University

Xiaolong Lu
Dalian Medical University

Xueting Wang
Dalian Medical University

Zhien Liu
Dalian Medical University

Liyang Guan
Dalian Medical University

Xin Li
Dalian Medical University

Zaixing Du
Dalian Medical University

Hang Ren
Dalian Medical University

Jihong Wang
Liaoning Normal University

Zunchun Zhou
Liaoning Ocean and Fisheries Science Research Institute

Li Lv (lv_li@126.com)
Dalian Medical University

Research Article

Keywords: Hepatocellular carcinoma, rAj-Tspin, ITGB1, ZYX, EMT, apoptosis

Posted Date: August 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3248897/v1
Abstract

rAj-Tspin, a soluble gene recombinant peptide from Apostichopus japonicus, can inhibit the integrin β1 (ITGB1)/FAK/AKT signaling pathway in hepatocellular carcinoma (HCC) via cell epithelial–mesenchymal transition (EMT) and apoptosis. Zyxin (ZYX) is a focal adhesion protein that is considered a novel mediator of EMT and apoptosis. However, the inhibitory mechanisms of rAj-Tspin in HCC and whether it is related to ZYX are unclear. We examined the antitumor effect of rAj-Tspin on the Huh7 human HCC cell line and on nude mouse models with subcutaneous injection or orthotopic intrahepatic transplantation of Huh7 cells. Our results show that rAj-Tspin strikingly reduced cell viability and promoted apoptosis in Huh7 cells and inhibited HCC tumor growth in nude mice. rAj-Tspin dose-dependently inhibited ITGB1 and ZYX protein expression in vivo and in vitro. Mechanistically, the FAK/AKT signaling pathway and the proliferation and invasion of HCC cells were suppressed upon ITGB1 and ZYX knockdown. Moreover, the effect of ITGB1 overexpression on the growth of HCC cells could be inhibited by rAj-Tspin. In contrast, the promoting effect of ITGB1 overexpression could be inhibited by ZYX knockdown. ZYX knockdown had no effect on ITGB1 expression. These findings suggest that ZYX is required for the indispensable role of ITGB1 in rAj-Tspin-alleviated HCC and provide an important therapeutic target for HCC. In summary, the anti-HCC effect of rAj-Tspin potentially involves the regulation of the ITGB1/ZYX/FAK/AKT pathway, which in turn impacts EMT and apoptosis.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide[1]. Increasing incidence and mortality from HCC poses a huge challenge to global healthcare. Patients with HCC have an unfavorable prognosis, and intrahepatic and extrahepatic metastases often occur due to its aggressive features[2]. Significant progress has been made in the treatment of HCC with the application of new first- and second-line agents; however, their efficacy is limited, and they are only effective for some patients or only prolong survival by several months[3]. As such, new therapeutic agents and targets are urgently needed to improve outcomes in patients with HCC.

rAj-Tspin is a soluble gene recombinant peptide from Apostichopus japonicus (A. japonicus). A. japonicus belongs to the phylum echinoderm and has a variety of biological activities[4]. Bioinformatics analysis of A. japonicus sequences using cDNA libraries revealed a high abundance of “A disintegrin and metalloproteinase with thrombospondin motifs”. Further analysis of the sequence through the EXPASY website revealed that it contained 10 thrombin sensitive protein-1 (TSP-1) domains. The 168-bp cDNA sequence of the third TSP-1 domain in arginine-glycine-aspartic acid (RGD) mode was synthesized, cloned and expressed, and the 6975.53-Da recombinant peptide obtained by expression was named rAj-Tspin. Growing evidence suggests that animal-derived RGD motile active peptides, namely, disintegrin, can competitively bind to integrin receptors, thereby blocking integrin-related signaling pathways, preventing the invasion and adhesion of tumor cells, and even promoting the apoptosis of tumor cells[5, 6]. We have previously shown that rAj-Tspin contributes to ITGB1/FAK/AKT in BEL-7402 HCC cells. Therefore, rAj-Tspin undoubtedly has therapeutic potential in the clinical treatment of HCC[7].
The importance of EMT and apoptosis in cancer treatment is highlighted by recent findings regarding the association of EMT and apoptosis with tumor progression and drug response[8]. It has been suggested that zyxin (ZYX), a focal adhesion protein for the regulation of EMT and apoptosis, participates in multiple cancers, including colon cancer, breast cancer, prostate cancer, and lung cancer[9–11]. However, the relevant mechanism and whether it participates in HCC are unclear. Focal adhesions (FAs) are cell-matrix contacts formed by a nascent adhesion complex composed of integrins and numerous other focal adhesion molecules, such as talin, paxillin, zyxin, VASP, and FAK[12]. In particular, ITGB1 is widely reported in hepatocellular carcinoma and plays an indispensable role in focal adhesion[13]. However, the molecular mechanism by which rAj-Tspin affects ZYX and ITGB1 to alleviate HCC remains unclear.

In the present study, we used the Huh7 human HCC cell line in vitro and constructed in vivo nude mouse models by subcutaneous and orthotopic intrahepatic transplantation of Huh7 cells to investigate whether inhibition of ZYX and ITGB1 by rAj-Tspin can inhibit EMT and apoptosis-mediated HCC by decreasing the phosphorylation level of FAK/AKT.

2. Materials and Methods

2.1 Cell culture

The human HCC cell line Huh7 and the immortalized human liver cell line LO2 were maintained in high glucose Dulbecco’s modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (HyClone). All cells were donated by the College of Life Sciences at Liaoning Normal University (Dalian, China) and grown at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2 Subcutaneous and orthotopic intrahepatic transplantation model

For generation of the subcutaneous transplantation model, 1 × 10⁷ Huh7 cells were detached and suspended in 100 μL serum-free DMEM/Matrigel (9:1). The cells were subcutaneously injected into BALB/c-nude mice (male, 5 weeks old, n = 8 per group). For generation of the orthotopic intrahepatic transplantation model, 1 × 10⁶ Huh7 cells in 20 µL serum-free DMEM were injected into each BALB/c-nude mouse. Through a 1 cm transverse incision in the upper abdomen under anesthesia, the cells were injected into the left hepatic lobe of each mouse (male, 5 weeks old, n = 6 per group). Mice were randomly divided into the saline group, rAj-Tspin (50 μg/kg) group, rAj-Tspin (100 μg/kg) group and rAj-Tspin (200 μg/kg) group, and the treatments were administered i.p. twice daily. The body weight of nude mice and the larger diameter of the tumor a (mm) and the smaller diameter b (mm) were measured and recorded every other day, and tumor volume was calculated as V = a × b²/2. All mice were sacrificed after 2 weeks, and the tumors and livers were collected, fixed with neutral formalin and prepared for histological examination. All mice were treated and housed according to the Feeding and Management of Experimental Animals of Dalian Medical University.
2.3 Histopathological staining

The liver tissues of the mice were embedded in paraffin to produce 5-µm thick sections. Tissue slices were stained with hematoxylin and eosin (H&E) to observe the tissue structure. An optical microscope (OLYMPUS) was used to detect the pathological changes and target protein expression in each tissue section.

2.4 Cell viability assay

Huh7 cells were seeded on 96-well plates (5 × 10^3 cells/well) 12–16 h, treated with rAj-Tspin at various concentrations for 24 h, 48 h, 72 h, and the cells were further incubated with 10% CCK-8 solution (Bioss) at 37°C for 2 h. This was followed by measuring the absorbance at 450 nm using a microplate reader (TECAN).

2.5 Small interfering RNA and plasmid transfection

Transfection with ZYX/ITGB1 small interfering RNA (siRNA; GenePharma) and ITGB1-overexpressing plasmid (GenePharma) was performed using Mate (GenePharma) according to the standardized protocol. ZYX siRNA: 5′-GUGUUACAAGUGUGAGGACTT-3′. ITGB1 siRNA: 5′-GCGAGTTGATATATTGAA-3′. siRNA was used to suppress ZYX/ITGB1 expression in Huh7 cells, and a recombinant plasmid construct of the gene was used to achieve the overexpression of ITGB1 in Huh7 cells.

2.6 Cell migration assay

Huh7 cells were seeded on 6-well plates (2 × 10^5 cells/well) for 24 h, wounded by scratching with sterile plastic 200-µL micropipette tips, and photographed using an inverted microscope (GROUPCR). After treatment with rAj-Tspin (0, 0.2, 0.4 and 0.8 µM) for 24 h or transfection, the culture wells were photographed again.

2.7 Cell invasion assay

Transwell chambers (Costar) were precoated with 50 µL serum-free DMEM/Matrigel (9:1) Matrigel matrix to facilitate the assessment of cell migration. The pretreated Huh7 cells (1 × 10^4) in 200 µL of serum-free medium were plated in the upper chamber, and 700 µL of culture medium with 10% FBS was added to the lower chamber. After a 24-h incubation, the migrated cells were fixed with 4% paraformaldehyde (Servicebio) and stained with 0.3% crystal violet (Solarbio). Three fields were selected at random through photography, and their numbers were documented.

2.8 Cell adhesion assay

For this assay, 96-well plates were precoated with 50 µL of 10 µg/mL fibronectin (FN, Solarbio) and sealed with 1% Bull Serum Albumin (BSA, Solarbio) at 37 °C for 1 h to facilitate the assessment of cell adhesion. The pretreated Huh7 cells (5 × 10^3) in 100 µL were seeded on 96-well plates. After a 2-h incubation, the cells were fixed and stained with 0.3% crystal violet. Three fields were selected at random
and photographed. The dye was extracted with 33% acetic acid, and the absorbance at 600 nm was
detected by a microplate reader.

2.9 TUNEL assay

Huh7 cells were seeded on 24-well plates (1 × 10⁴ cells/well) for 24 h, treated with rAj-Tspin (0, 0.2, 0.4
and 0.8 µM) for 24 h or transfected. After fixation with 4% paraformaldehyde and permeabilization with
0.3% Triton X-100 (Solarbio), the cells were incubated with TUNEL solution (Beyotime) for 2 h in the dark.
The nuclei were stained with DAPI (Solarbio), and three fields were selected at random through
photography.

2.10 Western blotting

Total protein was extracted from tissues or cells using RIPA buffer (Beyotime) containing PMSF
(Beyotime). The lysate was then sonicated and centrifuged at 4°C at 12000 r/min for 10 min. The
samples were added to 2× loading buffer followed by a denaturation step at 100°C. All proteins were run
on 8 – 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; concentrated gel, 80
V; separator gel, 120 V) and transferred to PVDF membranes (Millipore). Afterward, the membrane was
blocked with 10% skim milk at room temperature for 2 h and incubated overnight at 4°C with primary
antibodies against the following targets: ITGB1 (ab183666, Abcam), FAK (3285s, CST), p-FAK (3283s,
CST), AKT (10176-2-AP, Proteintech), p-AKT (28731-1-AP, Proteintech), ZYX (10330-1-AP, Proteintech), N-
cadherin (22018-1-AP, Proteintech), E-cadherin (20874-1-AP, Proteintech), Vimentin (10366-1-AP,
Proteintech), Bax (60267-1-Ig, Proteintech), Bcl-2 (12789-1-AP, Proteintech), cleaved caspase-3
(abs132005, Absin), and GAPDH (10494-1-AP, Proteintech). After several washings, the membrane was
then incubated with anti-mouse IgG or anti-rabbit IgG for 2 h and detected using ECL reagent (Meilunbio)
and an imaging system (Tanon). Quantification of the blots was performed by ImageJ software.

2.11 Coimmunoprecipitation (Co-IP) assay

Total protein was extracted from cells using lysis buffer. The relevant antibodies and protein A/G beads
were added to the protein lysates. Afterward, the lysates were incubated at 4°C overnight. The samples
were then subjected to SDS–PAGE electrophoresis by adding 1× loading buffer followed by a
denaturation step at 100°C.

2.12 Immunofluorescence

Huh7 cells were seeded on 24-well plates (1 × 10⁴ cells/well) at a confluence of 60%-80%. After fixation
and permeabilization, the cells were incubated with primary antibodies at 4°C overnight, labeled with
secondary antibodies and counterstained with DAPI. Images were acquired using a reverse fluorescence
microscope.

2.13 Bioinformatics analysis

Gene Expression Profiling Interactive Analysis (GEPIA, URL: http://gepia.cancer-pku.cn) was conducted to
analyze the expression of ZYX in HCC tumor tissue and normal tissue. The University of Alabama at
Birmingham (UALCAN, URL: http://ualcan.path.uab.edu) conducted an online analysis of Kaplan–Meier plots related to the ZYX gene in HCC patients.

2.14 Statistical analysis

Data analysis was performed using SPSS 22.0 software. All data are expressed as the mean ± standard deviation (SD). The two groups of data were compared using the independent sample t test, and comparisons of multiple variables were determined by one-way analysis of variance. Statistical analysis was performed using GraphPad Prism 8.4.3 software to draw column graphs. Statistical significance was defined as P < 0.05.

3. Results

3.1 rAj-Tspin inhibited the growth of subcutaneous tumors and orthotopic intrahepatic tumors derived from Huh7 cells

To evaluate the effectiveness of rAj-Tspin on HCC growth in vivo, we subcutaneously and intrahepatically inoculated Huh7 cells into nude mice (Figure 1A). We observed that the administration of rAj-Tspin resulted in a dose-dependent reduction in both the volume and weight of xenograft tumors but had no impact on the body weight of mice (Figure 1B-E). In addition, we performed HE staining on the xenograft tumors, further demonstrating the antitumor effect of rAj-Tspin in vivo (Figure 1F). In the orthotopic intrahepatic transplantation model, we observed a dose-dependent reduction in tumor area, size and number of nodules upon administration of rAj-Tspin (Figure 1G). This was further confirmed by H&E staining. Furthermore, we found that the rAj-Tspin-treated group had significantly decreased serum LDH levels without any effect on mouse body weight (Figure 1H, I).

3.2 rAj-Tspin targeted ITGB1 and inhibited the high expression of ZYX and ITGB1 in vivo

rAj-Tspin showed a strong affinity with ITGB1 domain and the potential binding sites are predicted to be the residues Arg43, Arg48, and Cys55 (Figure 2A). GEPIA revealed ZYX overexpression in HCC patient samples vs. normal samples (Figure 2B, P < 1e-12). We investigated the association between high ZYX expression and patient survival by using UALCAN and found a significant correlation with inferior prognosis (Figure 2C, P=0.002). Western blotting showed higher ZYX and ITGB1 expression in liver cancer tissue than in normal tissue (Figure 2D). Furthermore, rAj-Tspin dose-dependently attenuated the expression of ITGB1 and ZYX as well as the phosphorylation of FAK and AKT in vivo (Figure 2E, F).

3.3 rAj-Tspin repressed the growth of Huh7 cells in vitro

To evaluate the impact of rAj-Tspin on Huh7 cells, we exposed them to varying concentrations of rAj-Tspin for 24, 48, and 72 h using the CCK-8 assay. We observed a reduction in cell viability that was dependent on the dosage administered (Figure 3A). By cell scratch assay, Transwell assay and adhesion assay, as the rAj-Tspin concentration increased, the cellular migration, invasion and adhesion of Huh7 cells were decreased (Figure 3B). The TUNEL assay indicated that rAj-Tspin could promote apoptosis in
Huh7 cells in a dose-dependent manner (Figure 3C). The Western blot assay revealed that the expression levels of ZYX and ITGB1 were higher in Huh7 cells than in LO2 cells (Figure 3D). Similarly, the administration of rAj-Tspin induced a dose-dependent reduction in the expression levels of ITGB1 and ZYX as well as the phosphorylation levels of FAK and AKT \textit{in vitro} (Figure 3E). To further investigate the effect of rAj-Tspin on HCC apoptosis and EMT, we conducted Western blot analysis of apoptosis- and EMT-related proteins. The results showed that cleaved caspase-3 and Bax levels were increased, the Bcl-2 level was decreased, the N-cadherin and Vimentin levels were decreased and the E-cadherin level was increased in HCC cells treated with rAj-Tspin (Figure 3F). Thus, rAj-Tspin induced apoptosis and inhibited EMT in Huh7 cells. Collectively, these findings suggest that rAj-Tspin represses the growth of Huh7 cells through EMT and apoptosis \textit{in vitro}.

\subsection*{3.5 Knockdown of ITGB1 suppressed Huh7 cell progression \textit{in vitro}}

To explore the potential biological function of ITGB1, we transfected Huh7 cells with ITGB1 siRNA. The migration, invasion and adhesion capacities (Figure 4A) were significantly reduced in Huh7 cells transfected with si-ITGB1. The TUNEL assay indicated that ITGB1 knockdown could promote apoptosis in Huh7 cells (Figure 4B). The Western blot assay revealed that silencing ITGB1 suppressed the expression of ZYX and the phosphorylation of FAK and AKT (Figure 4C). Simultaneously, ITGB1 knockdown influenced the expression of apoptosis- and EMT-related proteins (Figure 4D), inhibited EMT and induced apoptosis in Huh7 cells. These results indicated that ITGB1 plays an important role in suppressing features related to disease progression in HCC cells \textit{in vitro}.

\subsection*{3.6 rAj-Tspin suppressed cell growth through ITGB1 inhibition in HCC cells}

To further clarify the mechanism underlying whether ITGB1 is a potential trigger in rAj-Tspin-mediated suppression of cell growth, we established ITGB1-overexpressing cells. The overexpression of ITGB1 enhanced the proliferation, migration and adhesion ability of Huh7 cells, while rAj-Tspin intervention attenuated this effect (Figure 5A). As expected, Western blot assays revealed that the overexpression of ITGB1 induced the accumulation of ZYX, increased the phosphorylation of FAK and AKT, and was effectively abolished by rAj-Tspin (Fig. 5B). Moreover, overexpression of ITGB1 suppressed apoptosis and triggered EMT in Huh7 cells, which was counteracted by rAj-Tspin treatment (Fig. 5C). In summary, these results demonstrate that the downregulation of ITGB1 is required for rAj-Tspin-mediated suppression of HCC growth.

\subsection*{3.7 Knockdown of ZYX suppressed Huh7 cell progression \textit{in vitro}}

To explore the specific role of ZYX in HCC cells, we transfected Huh7 cells with ZYX siRNA. The migration, invasion and adhesion capacities (Figure 6A) were significantly reduced in Huh7 cells transfected with si-ZYX. The TUNEL assay indicated that ZYX knockdown could promote apoptosis in Huh7 cells (Figure 6B). The Western blot assay revealed that silencing ZYX impeded the phosphorylation of FAK and AKT, while ITGB1 expression remained unaffected (Figure 6C). Simultaneously, ZYX knockdown could influence the expression of apoptosis- and EMT-related proteins (Figure 6D), inhibit EMT and induce
apoptosis of Huh7 cells. These results indicated that ZYX operates downstream of ITGB1 and plays important roles in suppressing the \textit{in vitro} progression of HCC cells.

\textbf{3.8 ZYX interacts with ITGB1 to facilitate HCC development}

We cotransfected si-ZYX and pcDNA-ITGB1 into Huh7 cells to explore the potential interaction between ZYX and ITGB1. Functionally, ZYX knockdown attenuated the promotion of HCC cell migration, invasion and adhesion by ITGB1 (Figure 7A). For further relative mechanistic research, western blot analysis revealed that ITGB1 increased the expression of ZYX and the phosphorylation of FAK and AKT, which were all reversed by ZYX knockdown (Figure 7B). Moreover, overexpression of ITGB1 suppressed apoptosis and triggered EMT in Huh7 cells, which was counteracted by ZYX knockdown (Figure 7C). Our coimmunoprecipitation data further confirmed that ZYX could indeed bind with ITGB1 (Figure 7D). By immunofluorescence staining, the colocalization of ITGB1 and ZYX was observed in Huh7 cells (Figure 7E). Overall, these results indicate that ZYX may promote HCC progression by interacting with ITGB1 via the FAK/AKT signaling pathway.

\textbf{Discussion}

In this study, we demonstrated for the first time the inhibitory effect of rAj-Tspin on an orthotopic intrahepatic transplantation model, and we investigated the roles and mechanisms involved in the regulation of ITGB1/ZYX in response to rAj-Tspin treatment in HCC cells. Our data suggest that rAj-Tspin provokes apoptosis and inhibits EMT by suppressing the FAK/AKT pathway. We found that rAj-Tspin decreased ITGB1 and ZYX expression, contributing to HCC cell apoptosis and tumor suppression. To our knowledge, our findings reveal a novel mechanism of rAj-Tspin-mediated HCC suppression with a focus on the role of ITGB1 and ZYX in FAK/AKT pathway induction.

HCC is a prevalent and severe malignancy\cite{14}. Radical hepatectomy remains the preferred therapeutic option for patients diagnosed with HCC in current clinical practice\cite{15, 16}. However, the long-term prognosis remains unsatisfactory, with a high recurrence rate of 60\% to 70\% within five years post-surgery\cite{17}. Therefore, the exploration of novel therapeutic drugs and targets is crucial for transforming the current state of medical treatment.

rAj-Tspin is a small polypeptide derived from \textit{A. japonicus} that contains RGD modules. As a targeting ligand, RGD offers significant advantages in terms of targetability and safety\cite{18}. Peptides containing the RGD domain have been demonstrated to exhibit specific recognition of integrin receptors, rendering them a precise therapeutic option for various tumors\cite{19}. In the present study, our results indicated that rAj-Tspin can significantly inhibit the growth of axillary tumors and in situ tumors in nude mice.

HCC is a prevalent malignancy worldwide and is characterized by high recurrence rates and a poor prognosis due to the highly proliferative and invasive nature of HCC cells\cite{20}. Loss of contact inhibition in cancer cells hinders apoptosis and promotes unlimited proliferation\cite{21}. Various apoptosis markers, such as Bcl2, Bax and cleaved caspase-3, are associated with tumor progression. During the process of
metastasis, individual tumor cells undergo EMT and subsequently invade the bloodstream [22]. EMT is characterized by the phenotypic transformation of cells from an epithelial to a mesenchymal state. Various epithelial and mesenchymal markers, such as Vimentin, N-cadherin and E-cadherin, are associated with tumor progression. In our study, rAj-Tspin reduced proliferation by downregulating Bcl-2 and upregulating Bax and cleaved caspase-3 and suppressed invasion by downregulating N-cadherin and Vimentin and upregulating E-cadherin in HCC cells.

Integrin-mediated signal transduction has been observed to induce cytoskeletal rearrangement through focal adhesion, thereby promoting tumor cell proliferation, invasion, and metastasis in cancer[23]. The extracellular domain of integrin interacts with the ligand in ECM, subsequently triggering downstream signaling pathways, typically involving a series of phosphorylation events such as FAK and AKT[24, 25]. Compared with the expression of other integrin subtypes, the expression of ITGB1 is significantly higher in liver tumor tissues. Multiple studies have demonstrated that ITGB1 inhibition resulted in marked growth inhibition of HCC cells via apoptosis induction and EMT inhibition[26, 27]. Our previous study revealed that rAj-Tspin is able to inhibit the development of HCC by inhibiting the FAK/AKT and ITGB1 signaling pathways[7]. However, the detailed functions of ITGB1 in the pathogenesis of HCC have not been completely elucidated. Our study has shown that rAj-Tspin treatment decreases ITGB1 expression in a dose-dependent manner \textit{in vivo} and \textit{in vitro}, and the overexpression of ITGB1 can be inhibited by rAj-Tspin, implicating rAj-Tspin as a potential ITGB1 inhibitor. Moreover, manipulation of ITGB1 expression levels through knockdown or overexpression can modulate the FAK/AKT signaling pathway, EMT progression, and cellular apoptosis.

Generally, adhesion between cells and the extracellular matrix is a crucial factor for cancer cells[21]. Focal adhesion has been identified as the pivotal determinant among the various microenvironmental factors that influence drug resistance in cancer cells[28]. ZYX is a pivotal protein associated with EMT and apoptosis in focal adhesion. High expression of ZYX in cancer is closely correlated with more aggressive behavior, a lower apoptotic ratio and diminished overall survival[29]. First, ZYX was overexpressed in both HCC tissues and cell lines, and the administration of rAj-Tspin led to a dose-dependent reduction in ZYX expression both \textit{in vivo} and \textit{in vitro}. Then, after ZYX knockdown by specific interfering RNA, we discovered that ZYX mediated the proliferation and metastasis of HCC cells \textit{in vitro}. These results indicated that ZYX might act as an oncogene in HCC progression. Moreover, co-IP and immunofluorescence assays demonstrated the interaction and colocalization of ZYX with ITGB1. Our in-depth research also revealed that ITGB1 helped the activation of the FAK/AKT pathway, which was mediated by ZYX.

Taken together, the results of this study provide compelling evidence that ZYX is involved in the effects of rAj-Tspin treatment on HCC and that ITGB1 downregulation plays an inhibitory role in the FAK/AKT pathway by suppressing ZYX expression, resulting in cell death and tumor suppression. These findings offer innovative insights into the mechanism of action of rAj-Tspin, suggesting that rAj-Tspin is a potential therapeutic agent against HCC and highlighting the ITGB1/ZYX/FAK/AKT axis as a promising target for cancer treatment.
Abbreviations

AKT, Protein kinase B; BCA, Bicinchoninic acid; DMEM, Dulbecco's modified Eagle Medium; EMT, Epithelial–mesenchymal transition; FAK, Focal adhesion kinase; FBS, Fetal bovine serum; H&E, Hematoxylin and eosin; HCC, Hepatocellular carcinoma; ITGB1, Integrin β1; PBS, Phosphate-buffered saline; PVDF, Polyvinylidene difluoride; RGD, arginine-glycine-aspartic acid; rAj-Tspin, Recombinant Apostichopus japonicus rAj-Tspin polypeptide; SDS–PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ZYX, zyxin

Declarations

Author contributions

YC, XTW, JHW, ZCZ and LL designed the experiments. YC wrote the manuscript. YC, XTW, LYG, XL, ZXD, ZEL and HR performed the animal experiments. YC performed the Western blot assays. YC, XLL and LL edited the manuscript. YC generated statistical analysis and handled figure data.

Acknowledgments

This work was supported by the Liaoning Provincial Department of Education Service Local Project (grant number LJKFZ20220251).

Conflict of interests

The authors declare no competing financial interests.

References


Figures
Figure 1

Effects of rAj-Tspin inhibition on Huh7 subcutaneous grafts and orthotopic intrahepatic transplantation model in nude mice.

(A) Schematic diagram of xenografts in BALB/c nude mice by inoculating Huh7 cells that were respectively at their underarm and intrahepatic. Then xenografts were treated rAj-Tspin starting at the 7th
day after injection and they were sacrificed at the 21th day after injection. (B) Images (C) and weights of excised tumors in the control and rAj-Tspin (50, 100, 200 mg/kg) groups of nude mice, (D) volumes measured at the indicated time points, (E) and effects of rAj-Tspin on body weight. (F) H&E staining of excised tumors from nude mice, Scale bar=100 mm (100×) and 50 mm (200×), n = 8. **p<0.01. (G) Representative images and H&E staining of liver tissues from mice orthotopically inoculated with Huh7 cells, Scale bar=100 mm (100×) and 50 mm (200×). (H) The release of serum LDH in nude mice was measured by LDH assay. (I) Effects of rAj-Tspin on body weight in nude mice, n=6. **p<0.01.

Figure 2

rAj-Tspin targets ITGB1 to inhibit ITGB1/ZYX overexpression in HCC.

(A) The chemical structure of rAj-Tspin and docking conformation showed the interaction of the rAj-Tspin with the active site of ITGB1 through MOE software. (B) The expression of ZYX in normal tissues and liver cancer tissues in the GEPIA cancer database. (C) The relationship between ZYX expression and survival time of HCC patients in the UALCANCAN cancer database. (D) Immunoblotting of ITGB1 and ZYX in liver tissue. (E and F) Immunoblotting of ITGB1, ZYX p-FAK, FAK, p-AKT, and AKT in liver tissue after rAj-Tspin treatment, n=3. **p<0.01.
Figure 3

rAj-Tspin inhibited the growth and promoted the apoptosis of Huh7 cells.

(A) Measurement of cell viability upon rAj-Tspin treatment with CCK-8. (B) The migration, invasion and adhesion capacity of Huh7 cells treated with increasing concentrations (0, 0.2, 0.4, 0.8 mM) of rAj-Tspin for 24 h. (C) Apoptotic cells were examined by TUNEL assay, Scale bar = 50 mm. (D) Immunoblotting of ITGB1 and ZYX in LO2 and Huh7 cells. (E and F) Immunoblotting of ITGB1, ZYX, p-FAK, FAK, p-AKT, AKT, Bcl-2, Bax, C-caspase-3, N-cadherin, E-cadherin, Vimentin, and GAPDH.
Bcl-2, Bax, cleaved-Caspase-3, vimentin, N-cadherin, and E-cadherin in Huh7 cells treated with increasing concentrations (0, 0.2, 0.4, 0.8 mM) of rAj-Tspin for 24 h (n=3). *p<0.05, **p<0.01.

Figure 4

ITGB1 knockdown inhibited growth and promoted apoptosis in Huh7 cells.

Knockdown of ITGB1 in Huh7 cells by siRNA treated with or without 0.8 mM rAj-Tspin for 24 h. (A) The migration, invasion and adhesion capacity of Huh7 cells. (B) Apoptotic cells were examined by TUNEL assay. Scale bar=50 mm. (C and D) Immunoblotting of ITGB1, ZYX p-FAK, FAK, p-AKT, AKT, Bcl-2, Bax, cleaved-Caspase-3, vimentin, N-cadherin, and E-cadherin in Huh7 cells (n=3). #p<0.05, ##p<0.01; *p<0.05, **p<0.01.

Figure 5
**rAj-Tspin suppressed HCC development via ITGB1.**

pcDNA3.1 or pcDNA-ITGB1 was transfected into Huh7 cells treated with or without 0.8 mM rAj-Tspin for 24 h. (A) The migration, invasion and adhesion capacity of Huh7 cells. (B and C) Immunoblotting of ITGB1, ZYX p-FAK, FAK, p-AKT, AKT, Bcl-2, Bax, cleaved-Caspase-3, vimentin, N-cadherin, and E-cadherin in Huh7 cells (n=3). *p<0.05, **p<0.01; *p<0.05, **p<0.01.

![Image](image-url)

**Figure 6**

**ZYX knockdown inhibited the growth and promoted the apoptosis of Huh7 cells.**

Knockdown of ZYX in Huh7 cells by siRNA treated with or without 0.8 mM rAj-Tspin for 24 h. (A) The migration, invasion and adhesion capacity of Huh7 cells. (B) Apoptotic cells were examined by TUNEL assay. Scale bar=50 mm. (C and D) Immunoblotting of ITGB1, ZYX p-FAK, FAK, p-AKT, AKT, Bcl-2, Bax, cleaved-Caspase-3, vimentin, N-cadherin, and E-cadherin in Huh7 cells (n=3). ns p 0.05; #p<0.05, ##p<0.01; *p<0.05, **p<0.01.
Figure 7

The ITGB1/ZYX axis was validated in Huh7 cells.

pcDNA3.1 or pcDNA-ITGB1 was transfected into Huh7 cells with or without ZYX knockdown by siRNA for 24 h. (A) The migration, invasion and adhesion capacity of Huh7 cells. (B and C) Immunoblotting of ITGB1, ZYX, p-FAK, FAK, p-AKT, AKT, Bcl-2, Bax, cleaved-Caspase-3, vimentin, N-cadherin, and E-cadherin in Huh7 cells (n=3). (D) Coimmunoprecipitation (Co-IP) assays were conducted in Huh7 cells transfected with a vector containing flag-tagged ITGB1 and ZYX; IgG was used as a control. (E) Confocal microscopy scan of immunofluorescence staining showed that ITGB1 (green) colocalized with ZYX (red) in Huh7 cells. DAPI was used for nuclear staining. Scale bar=10 mm. #p<0.05, ##p<0.01; *p<0.05, **p<0.01.