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Knockout of Ajuba Attenuates the Growth and Migration of Hepatocellular Carcinoma Cells

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Keywords
Hepatocellular carcinoma · Ajuba · Growth · Migration · Knockout

Abstract
Ajuba has been found to be mutated or aberrantly regulated in several human cancers and plays important roles in cancer progression via different signaling pathways. However, little is known about the role of Ajuba in hepatocellular carcinoma (HCC). Here, we found an upregulation of Ajuba expression in HCC tissues compared with normal liver tissues, while a poor prognosis was observed in HCC patients with high Ajuba expression. Knockout of Ajuba in HCC cells inhibited cell growth in vitro and in vivo, suppressed cell migration, and enhanced the cell apoptosis under stress. Moreover, re-expression of Ajuba in Ajuba-deficient cells could restore the phenotype of Ajuba-deficient cells. In conclusion, these results indicate that Ajuba is upregulated in HCC and promotes cell growth and migration of HCC cells, suggesting that Ajuba could possibly be a new target for HCC diagnosis and treatment.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and fatal human cancers in the world, especially in developing countries including China [Chen et al., 2016; Siegel et al., 2016]. Despite intensive efforts to improve early diagnosis and develop novel therapeutic strategies, the overall survival of HCC patients remains poor [Chen et al., 2016; Siegel et al., 2016]. Therefore, it is critical to understand the underlying mechanisms of HCC initiation and progression in order to develop early diagnosis and effective treatment methods.

Ajuba is a member of the Ajuba family which includes Ajuba, LIMD1 (LIM domain containing protein 1), and WTIP (Wilms tumor 1 interacting protein), and this protein family contains a unique pre-LIM region in the N-terminus and 3 tandem LIM domains in the C-terminus [Schimizzi and Longmore, 2015]. More and more evidence indicated that Ajuba might play important roles in cancer progression [Schimizzi and Longmore, 2015]. Firstly, Ajuba has been found to be mutated in several human cancers, including esophageal squamous cell carcinoma, aggressive cutaneous squamous cell carcinoma, and head and neck squamous cell carcinomas [Gao et al.,

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2014; Pickering et al., 2014; Cancer Genome Atlas Network, 2015; Zhang et al., 2015; Sawada et al., 2016; Xu et al., 2019], although the function of Ajuba mutation in these cancers needs further investigation. Secondly, the expression of Ajuba is aberrantly regulated in multiple human cancers, and the abnormal expression of Ajuba plays important roles in cell proliferation and migration. For example, the expression level of Ajuba is frequently upregulated in colorectal cancer, breast cancer, gastric cancer, esophageal squamous cell carcinoma, and pancreatic cancer, and Ajuba overexpression promotes cancer cell proliferation and migration [Shi et al., 2016; Jia et al., 2017; Li et al., 2019; Xu et al., 2019; Zhang et al., 2019]. However, Ajuba was found to be downregulated in malignant mesothelioma, and Ajuba overexpression suppresses malignant mesothelioma cell proliferation [Tanaka et al., 2015], suggesting that Ajuba can be a tumor suppressor or an oncogene in different types of cancer. Thirdly, Ajuba interacts with several kinases and regulates their activity which is involved in critical signaling pathways and associated with cancer progresses. For instance, Ajuba was identified as Aurora A partner and positively regulated its kinase activity, which was required for mitosis of human cells [Hirota et al., 2003; Bai et al., 2014]. Ajuba was also identified as a negative regulator of the Hippo signaling pathway and Wnt signaling pathway by associating with LATS1/2 or by promoting GSK-3β-mediated β-catenin phosphorylation, respectively [Haraguchi et al., 2008; Das Thakur et al., 2010; Reddy and Irvine, 2013; Rauskolb et al., 2014]. Although Ajuba has been shown to play multiple roles in many cancer progressions, the role of Ajuba in HCC remains largely unknown.

In this study, we found that Ajuba is upregulated in HCC, and its overexpression promotes HCC cell growth and migration, suggesting that Ajuba could possibly be a new target for HCC diagnosis and treatment.

Materials and Methods

Cell Lines

Human HCC cell lines Huh7 and SMMC-7721 were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium with 10% FBS in a static CO_2 incubator (37°C, 5% CO_2).

Human HCC Samples

Human HCC tissues and paired peritumoral non-tumor liver tissues were obtained from Zhongshan Hospital (Shanghai, China). Tumor samples were snap-frozen immediately after surgical excision in liquid nitrogen and then stored in a −80°C freezer. The tissue microarrays (TMAs), which include 90 human HCC tissues and paired peritumoral non-tumor liver tissues after surgical excision, were purchased from Outdo Biotech Co., Ltd. (Shanghai, China). The tissues of the TMAs were not from the samples of Zhongshan Hospital but were collected by Outdo Biotech Co., Ltd.

Plasmids and Antibodies

Human Ajuba (NCBI Accession NC_000014.9) was cloned into pCMV-Myc vector to generate constructs encoding Myc-tagged Ajuba for generation of Ajuba reconstitution cells.

Anti-Ajuba (4897S) and anti-β-actin (ab8226) were purchased from CST (MA, USA) and Abcam (MA, USA), respectively.

Immunohistochemistry Staining

Immunohistochemistry staining of the human HCC TMAs was performed by Outdo Biotech Co., Ltd. Briefly, 90 pairs of human HCC tissues and peritumoral non-tumor liver tissues (as control tissues) were fixed on microarrays and incubated with anti-Ajuba antibody (1:100). Antibody reaction was visualized using a fresh substrate solution containing diaminobenzidine, and scoring was conducted according to the ratio and intensity of positive-staining cells. Final score was determined as follows: score 0–1, low expression; score 1–2, moderate expression; and score >2, high expression.

Generation of Ajuba-Deficient Cells

CRISPR-Cas9 system was used to generate Ajuba-deficient Huh7 and SMMC-7721 cells as described before [Zhao et al., 2018]. The guide RNA sequence which targets Ajuba, TTTGAGGCGCCGCGCTACGAAGG, was designed using the guide design tool (http://crispr.mit.edu/) and then constructed into a pX335-U6-Chimeric-BB-CBh-hSpCas9n vector which contained Cas9 and 2A-Puromycin cassette. The construct was transiently transfected into Huh7 and SMMC-7721 cells, respectively. Cells with transfection were cultured under puromycin pressure for 2 days and then diluted to different concentrations for clone selection. After 2 weeks, clones were picked and cultured in 24-well culture plates. When the confluence of clones reached 80%, part of the cells were collected for clone screening. Western blot was used to verify the loss of Ajuba in these clones, while PCR was used to amplify the genomic region targeted by guide RNA followed by DNA sequencing.

Cell Proliferation Assay

Huh7 and SMMC-7721 cells were diluted to 10 cells/μL in cell culture medium and added into 96-well culture plates (100 μL/well). A Cell Counting Kit-8 (Dojindo Laboratories, Japan) was used for cell proliferation assay and the OD value was read at 490 nm using a Microplate Reader (Bio-Rad, USA).

Colony Formation Assay

Colony formation assay was performed as mentioned before [Zhu et al., 2017]. Huh7 or SMMC-7721 cells were diluted and added into 6-well culture plates (500 cells/well). Colonies were formed after 2 weeks. Formed colonies were washed with phosphate buffered saline for 3 times followed by a paraformaldehyde (4%) fixation for 15 min. Colonies were then colored by crystal violet and quantified with ImageJ software.

Tumor Growth Assay

Six-week-old nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Ajuba wild type (WT), knockout (KO), and reconstitution (RES) Huh-7 cells were diluted to 1×10^6 cells/200 μL in PBS and subcutaneously injected into the right flanks of nude mice. A measurement of tumor volumes was performed every 3–4 days, and tumor weights were measured after mice were sacrificed 4 weeks later.

Migration Assay

Migration assay was performed as described before [Shao et al., 2018]. Huh7 or SMMC-7721 cells were diluted to 2×10^4 cells/200 μL in cell culture medium without FBS and added into the upper chamber of a 24-well transwell plate (Corning), and 800 μL cell culture medium with 10% FBS was added into the lower chamber. After 36 h, the non-migrated cells in the upper chamber were removed by a cotton bud, and migrated cells were fixed with paraformaldehyde (4%) for 30 min, followed by crystal violet staining. Five fields of vision were chosen randomly under the microscope and imaged. The number of migrated cells was quantified with ImageJ software.

Apoptosis Assay

Huh7 or SMMC-7721 cells were diluted and added into 6-well culture plates. Dulbecco's Modified Eagle's Medium without FBS was used for serum starvation treatment. After 48 h, cells were collected and the apoptosis level was analyzed by flow cytometry with a FITC Annexin-V Apoptosis Detection Kit (BD Biosciences).

Analysis of Expression and Prognostic Value of Ajuba

Ajuba was searched in the Gene Expression Profiling Interactive Analysis (GEPIA) database and the results of expression analysis and survival analysis were shown in Expression DIY item and Survival item, respectively. The expression information of Ajuba in HCC and normal tissue could be acquired by selecting LIHC dataset. The results of survival analysis could be acquired by selecting LIHC dataset and setting other options as default. The statistical analysis of comparing Ajuba expression levels between HCC and normal tissue was performed by ANOVA, and the p value was displayed. The comparison of overall survival between high and low Ajuba expression groups was performed by log-rank test, and the p value was displayed.

Statistical Analysis

Quantitative data are represented as mean ± SD. Unpaired t-test with Welch correction was used to compare groups; $p < 0.05$ was considered statistically significant.

Results

Ajuba Is Frequently Upregulated in HCC

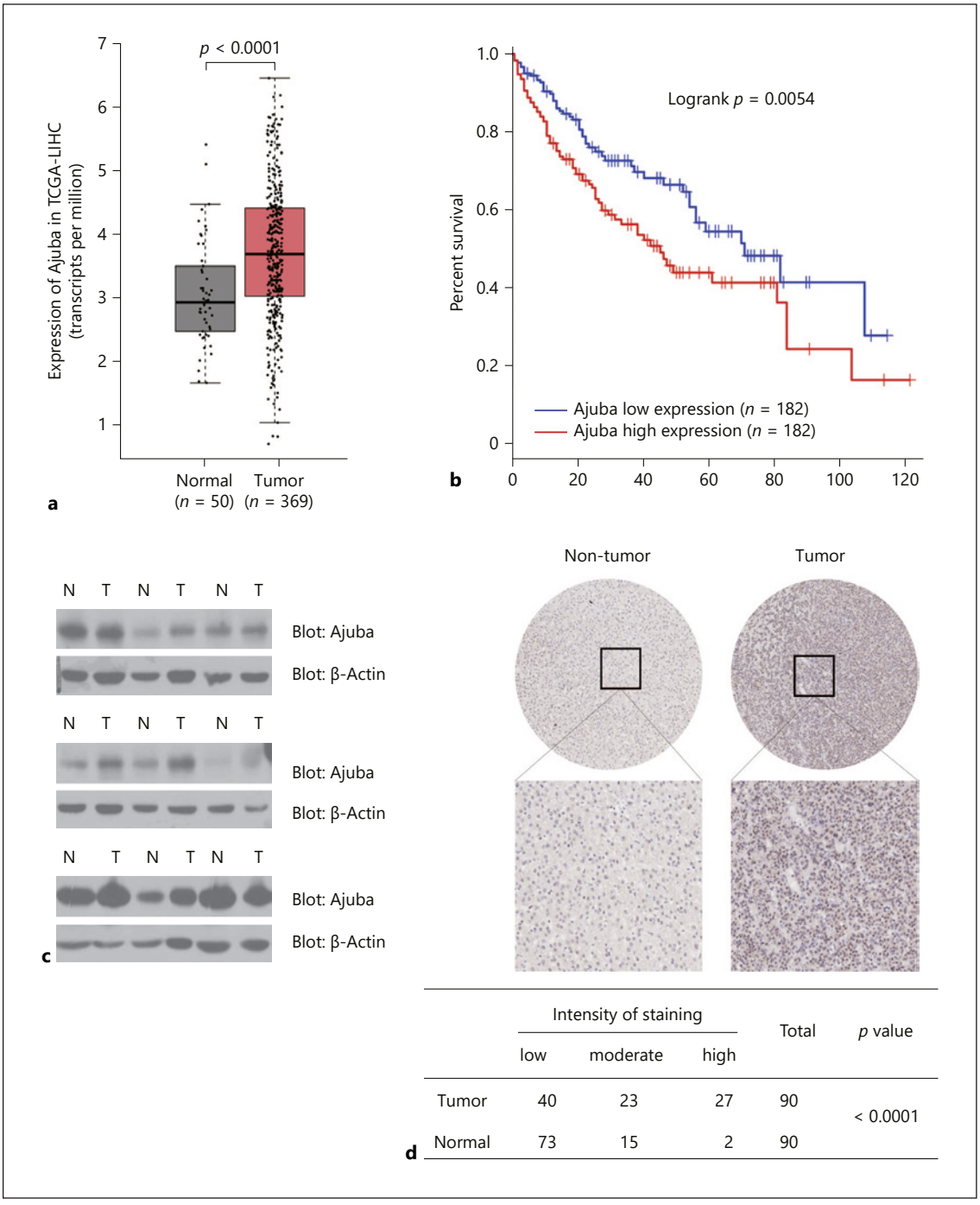
To investigate the expression level of Ajuba in HCC, we first analyzed the RNA-seq data from GEPIA database in Cancer Genome Atlas (TCGA) and found Ajuba is frequently upregulated in HCC tissues compared with the normal liver tissues (Fig. 1a). Moreover, Kaplan-Meier analysis revealed that high Ajuba expression in HCCs correlated with a worse overall survival of patients (Fig. 1b).

To further confirm that Ajuba is upregulated in HCC, we examined the protein expression level of Ajuba in paired human HCC tissue and their matched non-tumorous liver specimens by western blot or immunohistochemical staining using anti-Ajuba antibody. As shown in Figure 1c, the protein expression level of Ajuba was increased in 45% HCC tissues (4 out of 9) compared with the matched non-tumor liver tissues as analyzed by western blot. Similarly, immunohistochemical staining on a TMA containing 90 human HCC tissues and paired peritumoral non-tumor tissues showed that the expression level of Ajuba in human HCC tissues was significantly higher than in matched non-tumor tissues (Fig. 1d). These results indicate that the expression of Ajuba is upregulated in HCC, and Ajuba may be a valuable biomarker in HCC patients.

Knockout of Ajuba Attenuates HCC Cell Growth

The CRISPR-Cas9 system was used to generate Ajuba-deficient HCC cell lines in order to investigate the potential role of Ajuba in HCC. sgRNA was designed to target exon 1 of the Ajuba gene and was transfected into Huh7 and SMMC-7721 cells. Clones were screened, and the total loss of Ajuba protein in Ajuba-deficient Huh7 and SMMC-7721 cells was confirmed by western blot with an anti-Ajuba antibody (Fig. 2a). The deletion of some base pairs in Ajuba exon 1 which caused the frame-shift mutation was detected by sequencing (data not shown).

The effects of Ajuba deficiency on Huh7 and SMMC-7721 cells were examined by different approaches. First, the growth of wild type or Ajuba-deficient Huh7 and SMMC-7721 cells was analyzed by Cell Counting Kit-8 (CCK-8) assay. Compared with wild type cells, the growth of Ajuba-deficient Huh7 and SMMC-7721 cells was suppressed (Fig. 2b). The focus formation assay also showed that focus formation frequency of Ajuba-deficient Huh7 and SMMC-7721 cells was significantly decreased compared with control cells (Fig. 2c). In order to further confirm the role of Ajuba in HCC cell growth, Ajuba was reconstituted in Ajuba-deficient Huh7 cells, and the growth of Ajuba-reconstitution cells was analyzed by the CCK-8 assay and focus formation assay, respectively. As shown in Figure 2d, the expression level of Ajuba in Ajuba-deficient cells after reconstitution was similar to the endogenous Ajuba in wild type cells as examined by western blot. Re-expression of Ajuba could increase the growth and focus formation of Ajuba-deficient Huh7 cells (Fig. 2e, f), further suggesting that Ajuba plays important roles in the growth of HCC cells.



	Intensity of staining			Total	p value
	low	moderate	high		
Tumor	40	23	27	90	< 0.0001
Normal	73	15	2	90	

Fig. 1. Ajuba is upregulated in HCC. **a** The expression of Ajuba was measured by RNA-seq in the TCGA data set including 50 normal liver tissues and 369 HCC tissues. Ajuba expression in HCC tissues is higher than in normal tissues. **b** Survival rates based on RBM47 Ajuba were analyzed by Kaplan-Meier survival method in HCC patients. The half of patients with higher expression of Ajuba mRNA is indicated in red and that with lower expression is indicated in blue. A log-rank test was used to compare the variance between the 2 groups. **c** The protein level of Ajuba was examined in 9 pairs of HCC tumor tissues (T) and corresponding peritumor tissues (N) by western blot using anti-Ajuba antibody. β-Actin was used as loading control. **d** Two representative immunohistochemical staining images of a tissue array containing 90 HCC samples with anti-Ajuba antibody (upper panel). Statistical analysis of the immunohistochemistry results is shown below (Fisher exact test).

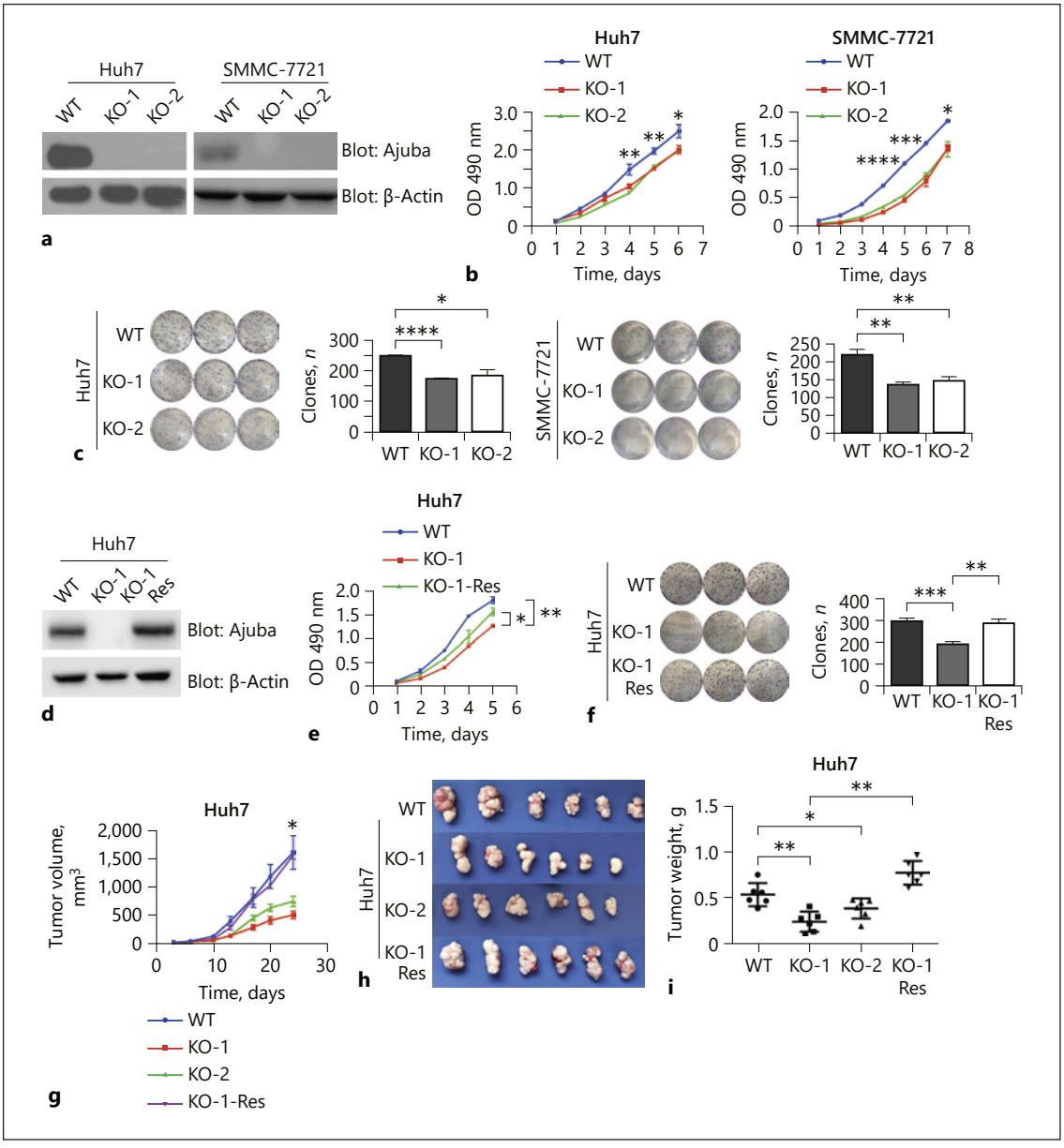


Fig. 2. Knockout of Ajuba attenuates the growth of HCC cells. **a** Ajuba-deficient Huh7 and SMMC-7721 cells were generated by CRISPR-CAS9 system. The Ajuba-deficient clones were identified by western blot using anti-Ajuba antibody. β-Actin was used as loading control. **b** CCK-8 assay was performed to determine the proliferation of Ajuba-deficient Huh7 and SMMC-7721 cells. **c** Effect of Ajuba deficiency on cell growth of Huh7 and SMMC-7721 cells was analyzed by colony formation assay. **d**–**f** Ajuba reconstitution rescued the growth of Ajuba-deficient Huh7 cells. The expression of Ajuba was examined by western blot. β-Actin was used as loading control (**d**). Representative results show that Ajuba reconstitution rescued the growth of Ajuba-deficient Huh7 cells as examined by CCK-8 assay (**e**) or colony formation assay (**f**). The experiments were repeated at least 3 times, and the histograms represent the mean numbers of colonies from triplicate tests. **g**–**i** Ajuba deficiency inhibited the growth of xenograft tumors derived from Huh7 cells. Xenograft tumors derived from Huh7 wild type, deficient, and reconstituted cells were measured by growth (**g**), tumor volume (**h**), and tumor weight (**i**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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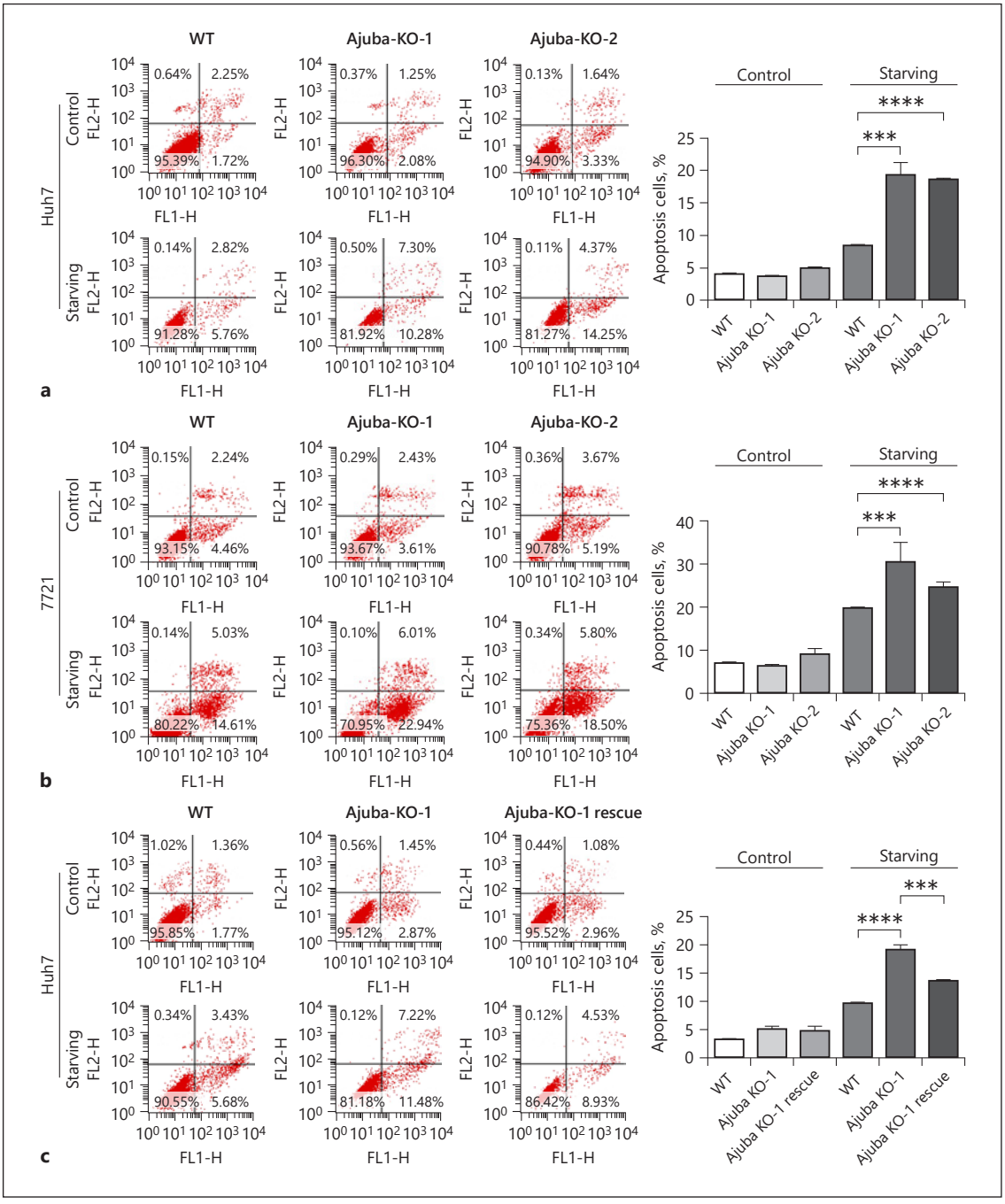

Fig. 3. Knockout of Ajuba enhances the apoptosis of HCC cells. **a**, **b** Ajuba deficiency enhances the apoptosis of Huh7 and SMMC-7721 cells. Huh7 (**a**) and SMMC-7721 (**b**) cells under normal or serum starvation conditions were analyzed by flow cytometry using an FITC-Annexin V apoptosis detection kit according to the manufacturer's instructions. Data represent mean ± SD from 3 independent experiments. **c** Ajuba reconstitution decreased the apoptosis of Ajuba-deficient Huh7 cells. The apoptosis rates of Huh7 wild type, deficient, and reconstituted cells under normal or serum starvation conditions were analyzed. Data represent mean ± SD from 3 independent experiments. *** $p < 0.001$; **** $p < 0.0001$.

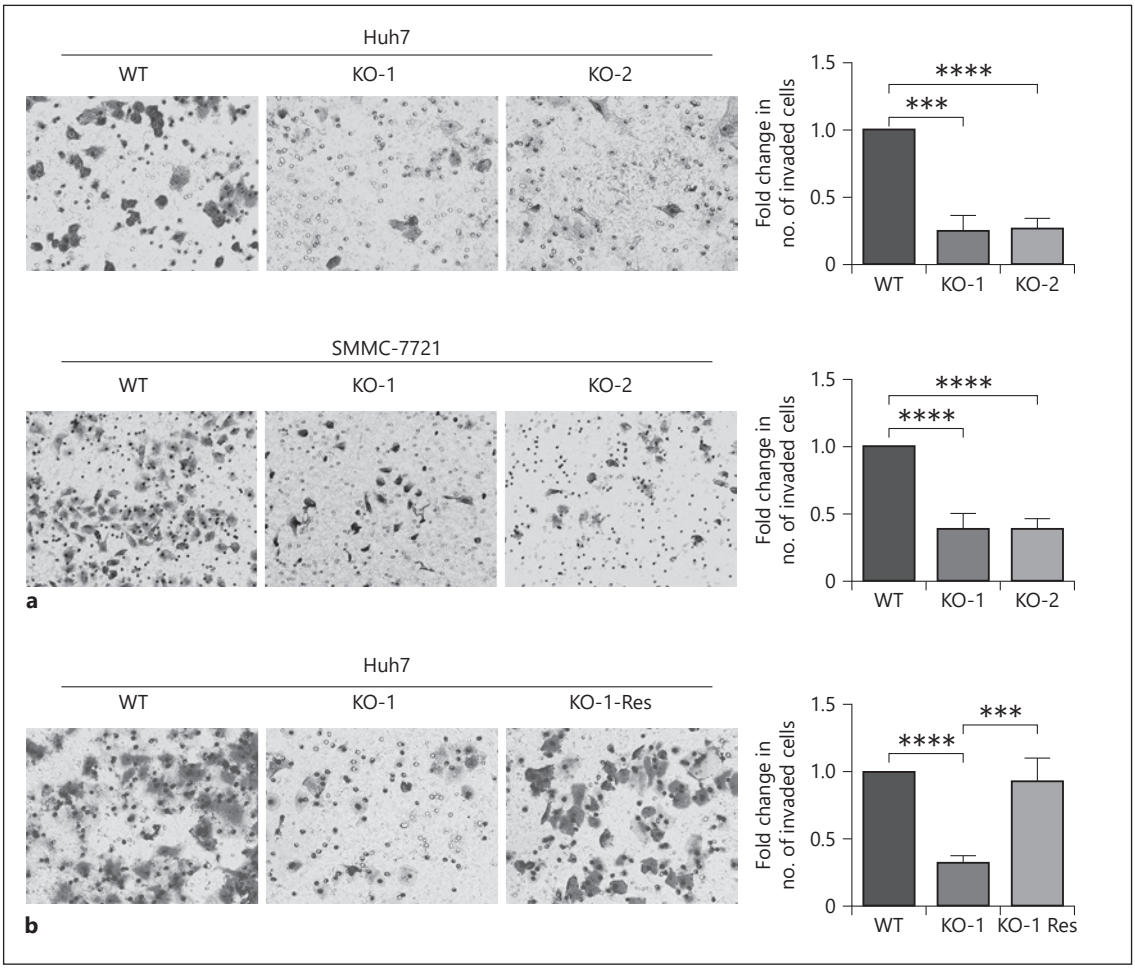

Fig. 4. Knockout of Ajuba attenuates the migration of HCC cells. **a** Ajuba deficiency suppressed the migration of Huh7 and SMMC-7721 cells. Representative images of migrated cells were stained with crystal violet and originally magnified ×100. The histograms show the mean numbers of migrated cells from 3 independent tests (mean ± SD). **b** Ajuba reconstitution increased the migration of Ajuba-deficient Huh7 cells. The migration of Huh7 wild type, deficient, and reconstituted cells was analyzed by trans-well assay. Data represent mean ± SD from 3 independent experiments. *** $p < 0.001$; **** $p < 0.0001$.

Next, the growth of wild type, Ajuba-deficient, and Ajuba-reconstitution Huh7 cells in vivo was examined by injecting subcutaneously into nude mice. Compared with tumors derived from control cells, the volume and weight of tumors derived from Ajuba-deficient cells were dramatically decreased, and re-expression of Ajuba in Ajuba-deficient cells could increase the tumor volume and tumor weight (Fig. 2g–i). These results indicated that knockout of Ajuba suppressed cell growth in culture and tumor growth in vivo, suggesting that Ajuba may function as an oncogene in HCC.

Knockout of Ajuba Enhances HCC Cell Apoptosis under Stress

FITC-Annexin V kit and flow cytometry were used to examine the apoptosis level of wild type and Ajuba-deficient HCC cells. As shown in Figure 3a and b, Ajuba knockout had no effects on cell apoptosis in HCC cells under normal conditions. However, Ajuba deficiency enhanced the Huh7 and SMMC-7721 cell apoptosis under serum starvation conditions compared with control cells. Moreover, re-expression of Ajuba could suppress the apoptosis of Ajuba-deficient Huh7 cells (Fig. 3c). Taken together, these results indicated that knockout of Ajuba enhanced the apoptosis of HCC cells under stress condition.

Knockout of Ajuba Attenuates HCC Cell Migration

To test whether Ajuba plays important roles in HCC cell migration, we performed cell migration assays using wild type and Ajuba-deficient Huh7 and SMMC-7721 cells. As shown in Figure 4a, the migration of Ajuba-deficient Huh7 and SMMC-7721 cells was significantly decreased compared with wild type cells, suggesting that Ajuba positively regulated HCC cell migration. Moreover, re-expression of Ajuba could increase the migration of Ajuba-deficient Huh7 cells (Fig. 4b). These results revealed that knockout of Ajuba decreased the migration of HCC cells.

Discussion

Although Ajuba was found to enhance the growth and migration of many cancer cells [Shi et al., 2016; Jia et al., 2017; Li et al., 2019; Xu et al., 2019; Zhang et al., 2019], Liu et al. [2018] recently reported that Ajuba inhibited the cell growth of HCC. They found that low Ajuba expression was a strong indicator for an inferior overall survival, although the expression level of Ajuba in HCC tissues was not analyzed in this study [Liu et al., 2018]. Interestingly, we found Ajuba is frequently upregulated in HCC tissues compared with the normal liver tissues by analyzing the RNA-seq data from GEPIA database in TCGA (Fig. 1a). The upregulation of Ajuba in HCC is consistent with its expression in many other cancers such as colorectal cancer, gastric cancer, breast cancer, pancreatic cancer, and esophageal squamous cell carcinoma [Shi et al., 2016; Jia et al., 2017; Li et al., 2019; Xu et al., 2019; Zhang et al., 2019]. Moreover, Kaplan-Meier analysis revealed that high Ajuba expression in HCCs correlated with a worse overall survival of patients (Fig. 1b), which is not consistent with the previous study [Liu et al., 2018]. Our study also revealed that knockout of Ajuba suppressed cell growth in culture and tumor growth in vivo, enhanced the apoptosis of HCC cells under stress condition, and decreased the migration of HCC cells. Taken together, we demonstrated that Ajuba was upregulated in HCC, and high levels of Ajuba expression correlated with a worse overall survival of patients with HCC. Moreover, Ajuba deficiency suppressed HCC cell growth and migration. These results suggest that Ajuba could possibly be a new target for HCC diagnosis and treatment.

Acknowledgment

We thank Outdo Biotech Co., Ltd (Shanghai, China) for providing the TMA samples and performing the immunohistochemistry staining.

Statement of Ethics

All patients involved in this study gave their informed consent. All experimental procedures were approved by the institutional review board of Fudan University.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Yichen Le, Jiaxue Wu, and Long Yu conceived and planned the experiments. Yichen Le, Meirong Bai, and Ying Wang carried out the experiments. Yi He contributed to the analysis of online databases. Yichen Le, Yi He, and Jiaxue Wu contributed to the interpretation of the results. Yichen Le took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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