ADAR1 p110 Enhances Adhesion of Tumor Cells to Extracellular Matrix in Hepatocellular Carcinoma via Up-Regulating ITGA2 Expression

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Data Collection B
Statistical Analysis C
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Background: Intrahepatic and distant metastases could be the major cause of treatment failure in hepatocellular carcinoma (HCC). The deep mechanism of HCC metastasis is closely related to the interaction between integrins and extracellular matrix (ECM) in tumor microenvironment.

Material/Methods: *In vitro* cell adhesion assay was performed to determine the capability of adhering to ECM elements of HCC cells. To modulate the expression status of ADAR1 p110 in tumor cells, lentivirus system was applied. Meanwhile, patients' HCC samples and orthotopic xenograft mouse model were used for verifying our *in vitro* data.

Results: ADAR1 p110 could strongly enhance the adhesion of HCC tumor cells to ECM, which was usually regarded as the initiation of tumor invasion. Such phenotype was caused due to up-regulation of ITGA2 both in mRNA and protein level. Moreover, specimen collected from HCC patients revealed a positive correlation between ADAR1 and ITGA2. Finally, ADAR1 p110 promoted HCC metastasis was verified when we applied orthotopic xenograft mouse model.

Conclusions: ADAR1 could enhance HCC metastasis by promoting tumor cells adhering to ECM via increasing ITGA2 expression. This phenomenon could provide novel information to better understanding the mechanism of HCC metastasis procedure.

MeSH Keywords: Adenosine Deaminase • Carcinoma, Hepatocellular • Cell Adhesion Molecules • Integrin alpha2

Abbreviations: HCC – hepatocellular carcinoma; ECM – extracellular matrix; ADAR1 – adenosine deaminases that act on RNA

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Background

Hepatocellular carcinoma, the major component of the primary liver cancers, is a highly malignant pathological pattern which made it the second leading cause for cancer-related death worldwide, and the scenario is even worse in Asia, especially in China [1,2]. Surgical treatments including liver resection and transplantation are recommended as the most curative therapy for early stage HCC patients. However, most patients were firstly diagnosed as late or advanced HCC and excluded for these procedures [3,4]. One of the reasons for this situation is early metastasis which usually happened intrahepatically [5]. Therefore, a better understanding for the deep mechanism of HCC invasion could be a key to develop effective approaches for its control.

A number of cells' biological behaviors are mediated by cell adhesion to extracellular matrix (ECM), including reorganization of cytoskeleton [6], cell growth [7] and importantly, cell mobility [8]. Among those cell surface adhesion proteins, integrin have been regarded as a strong cancer promoter via initiating metastasis and supporting proliferation [9,10]. Integrins are heterodimeric transmembrane proteins consisted by 2 subunits, namely alpha (α) and beta (β) [11,12]. According to previous studies, α subunit can associate with components that form ECM, and the β subunit is thought to participate in intracellular signal transduction, including AKT, ROCK, and FAK [13–15]. The way α and β subunits combine determines which ECM component is recognized. In normal hepatocytes, collagen IV is most commonly found and can interact with $\alpha1\beta1$ and $\alpha2\beta1$-integrin [16].

Local invasion of the host tissue and metastasis are hallmarks of cancer progression [17]. Invasion of tumor cells into the host tissue is regulated by the matrix microenvironment, mainly introduced by integrin family members [9,18]. Usually, integrin adhesion to the ECM provides the traction required for tumor cell invasion. Also, integrins assist tumor cell invasion via regulating the localization and activity of matrix-degrading proteases, such as matrix metalloprotease 2 (MMP2) and urokinase-type plasminogen activator (uPA) [19]. Among these reported features of integrins, the function of integrin $\alpha2$ (ITGA2) in different cancer types is controversially. ITGA2 can reverse some of the malignant properties of breast cancer [20], while promoting invasion and migration abilities in HCC [21].

Adenosine deaminases, which act on RNA (ADAR1), are regarded as the major cause of disrupted A to I editing balance in coding regions and noncoding Alu repetitive elements in human HCC [22]. Based on previous data, both ADAR1 and ADAR2 insert function during HCC progression, while ADAR1 has a stronger expression [23].

In this study, we first revealed ADAR1 can directly influence ITGA2 expression, and this can modulate the interaction between HCC tumor cells and ECM components. Finally, we showed that metastasis is promoted due to this ADAR1-ITGA2 axis.

Material and Methods

Cell culture

The LM3 cells were a gift received from Prof. Zheng Shusen [24], and the SK-Hep1 was obtained from ATCC (Manassas, VA). Both cell lines were maintained in DMEM cell culture medium (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine. All cell lines were cultured in a 5% (v/v) CO_2 humidified incubator at 37°C.

### Transfection and lentivirus infection

The overexpression plasmid for ADAR1 p110 pWPI-ADAR1-p110 and knocking-down (KD) plasmid for ADAR1 pLKO-ADAR1 were provided as gifts from Prof. Cai Xiujun [23]. Cells of stably expressing ADAR1 p110 or KD ADAR1 were established according to a previous report [25]. Briefly, the core plasmid (pWPI or pLKO) was transfected into 293T cells together with packaging plasmid (psPAX2) and envelope plasmid (pMD2.G). After 48 h, lentivirus supernatant was collected and frozen at –80°C for later infection.

Western blot analysis

RIPA lysis buffer was used to obtain total proteins (40 µg) from certain cells. Then, the proteins were separated by 8% SDS/PAGE gel and transferred onto PVDF membranes (Millipore, Billerica, MA). After membranes blocking, appropriate dilutions of specific primary antibodies were used to incubate the membranes, and the HRP-conjugated secondary antibodies ensured the blots could be detected by the ECL system (Thermo Fisher Scientific, Rochester, NY). Anti-GAPDH (1: 1000, 6c5) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ADAR1 (1: 1000, monoclonal, ab126745) antibody, ITGA2 (1: 1000, ab133557), and anti-ADAR1 (1: 1000, polyclonal, ab88574) antibody were purchased from Abcam (Shanghai, China).

Cell adhesion assay

For cell adhesion assays, 10^4 cells per well were seeded in matrigel (354234, BD Biosciences) fibronectin- (F2006, Sigma), laminin (L6274, Sigma)-, vitronectin (V8379, Sigma)-, or collagen IV (C7521, Sigma) -coated 24-well plates for 2–4 h. Given a certain period of time for cell adhesion (SK-Hep1 for 30 min
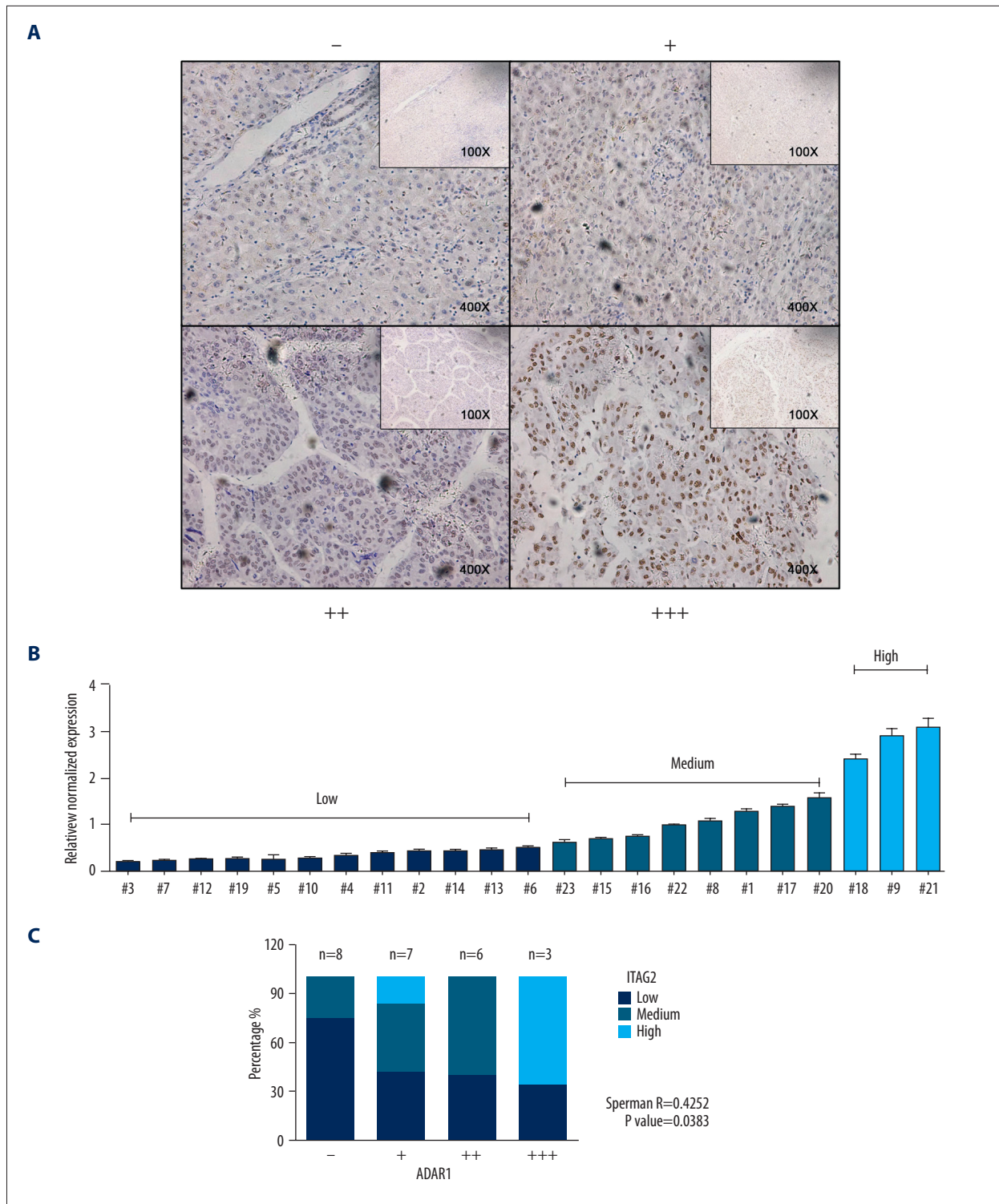
Figure 1. ITGA2 expression is positively correlated with ADAR1 in human HCC samples. (**A**) Representative images of ADAR1 IHC staining in human HCC samples. The levels were divided into 4 groups, namely −, +, ++ and +++. (**B**) We collected RNA from 23 human HCC samples from our hospital and performed RT-qPCR to detect expression level of ITGA2. Then we divided the results into 3 groups based on their relative normalized expression: low, 0–0.6; medium, 0.6–2.0; high, above 2.0. (**C**) We performed spearman correlation analysis for ADAR1 and ITGA2 according to our staining results. R=0.4252, p=0.0383. Data shown are mean ±SD.

and LM3 for 120 min), the wells were washed with PBS. After being fixed in 4% paraformaldehyde, the cells were stained with crystal violet, washed by PBS, and then dissolved in dimethylsulfoxide. OD value was then detected at 540 nm by an ELISA reader (Beckman). Alternatively, staining results could also be directly photographed.

For the function inhibition related assay, $5×10^4$ cells were incubated in culture medium including anti-integrin α2 (1: 100, AK7, Invitrogen) for 2 h at 37°C, then proceeding to next stage [26]. We used anti-GAPDH (1: 1000, 6c5, Santa Cruz) as a control antibody.

Quantitative real-time PCR analysis

We used TRIzol reagent (Invitrogen) to extract total RNA from either cultured cells or frozen tissue of HCC. Reverse transcription was performed by using Superscript III transcriptase (Invitrogen). To detect the mRNA expression level of a gene of interest, quantitative real-time PCR (qRT-PCR) was then performed using the Bio-Rad CFX96 system (Bio-Rad, Hercules, CA) with SYBR green. After that, expression levels of certain genes were normalized to GAPDH. The primers used were: ITGA1 F' CTGGACATAGTCATAGTGCTGGA, R' ACCTGTGTCTGTTTAGGACCA; ITGA2 F' CCTACAATGTTGGTCTCCCAGA, R' AGTAACCAGTTGCC TTTTGGATT; ITGA3 F' TGTGGCTTGGAGTGACTGTG, R' TCATTGCCTCGCACGTAGC; ITGA4 F' CACAACACGCTGTTCGGCTA, R' CGATCCTGCATCTGTAAATCGC; ITGA5 F' GGCTTCAACTT AGACGCGGAG, R' TGGCTGGTATTAGCCTTGGGT; ITGA6 F' CAGTGGAGCCGTGGTTTTG, R' CCACCGCCACATCATAGCC; ITGB1 F' CCTACTTCTGCACGATGTGATG, R' CCTTTGCTACGGTTGGTTACATT; ITGB2 F' AAGTGACGCTTTACCTGCGAC, R' AAGCATGGAG TAGGAGAGGTC; ITGB3 F' GTGACCTGAAGGAGAATCTGC, R' CCGGAGTGCAATCCTCTGG; ITGB4 F' CTCCACCGAGTCAGCCTTC, R' CGGGTAGTCCTGTGTCCTGTA.

**A**

B

SK-Hep1

C

LM3
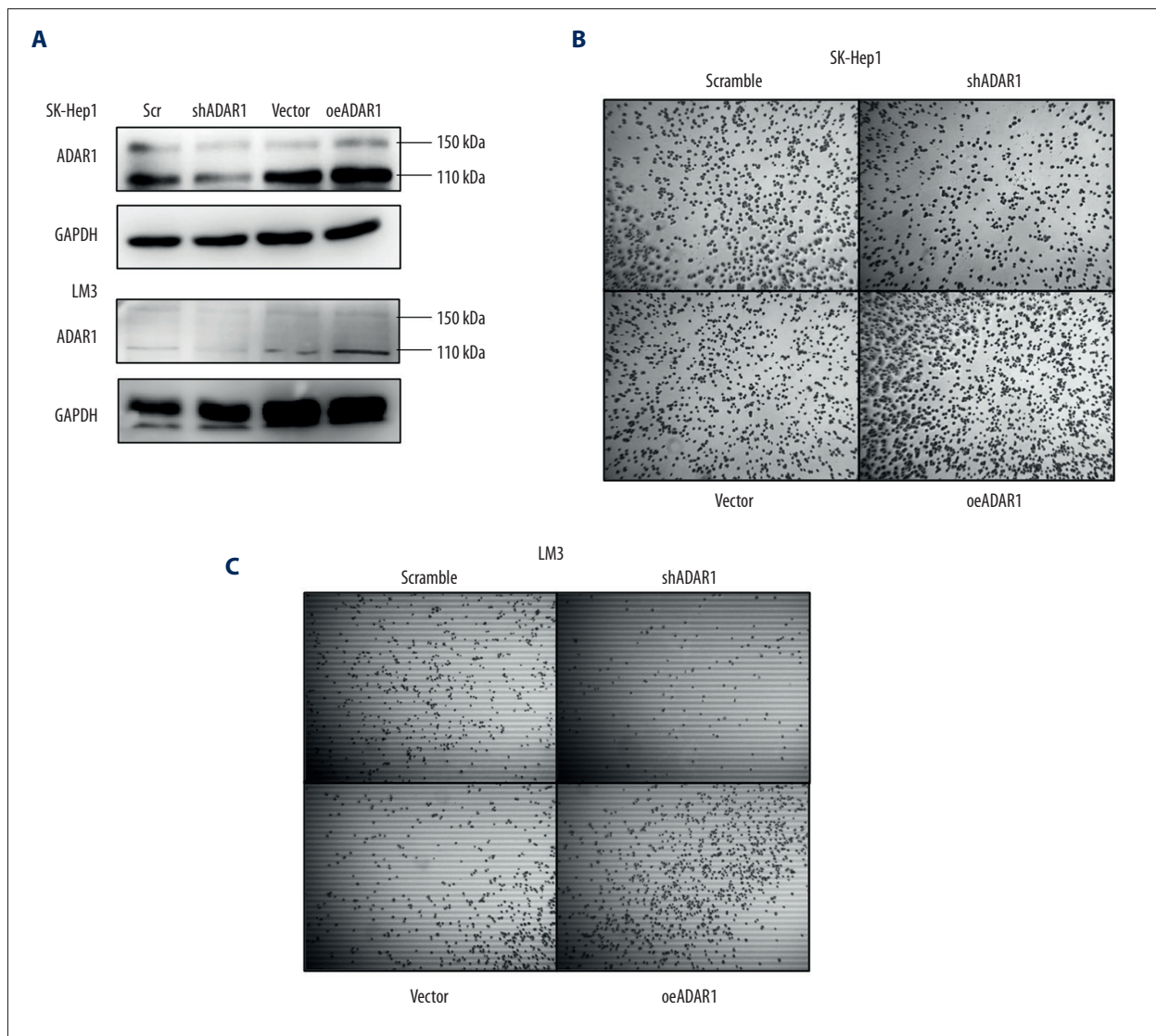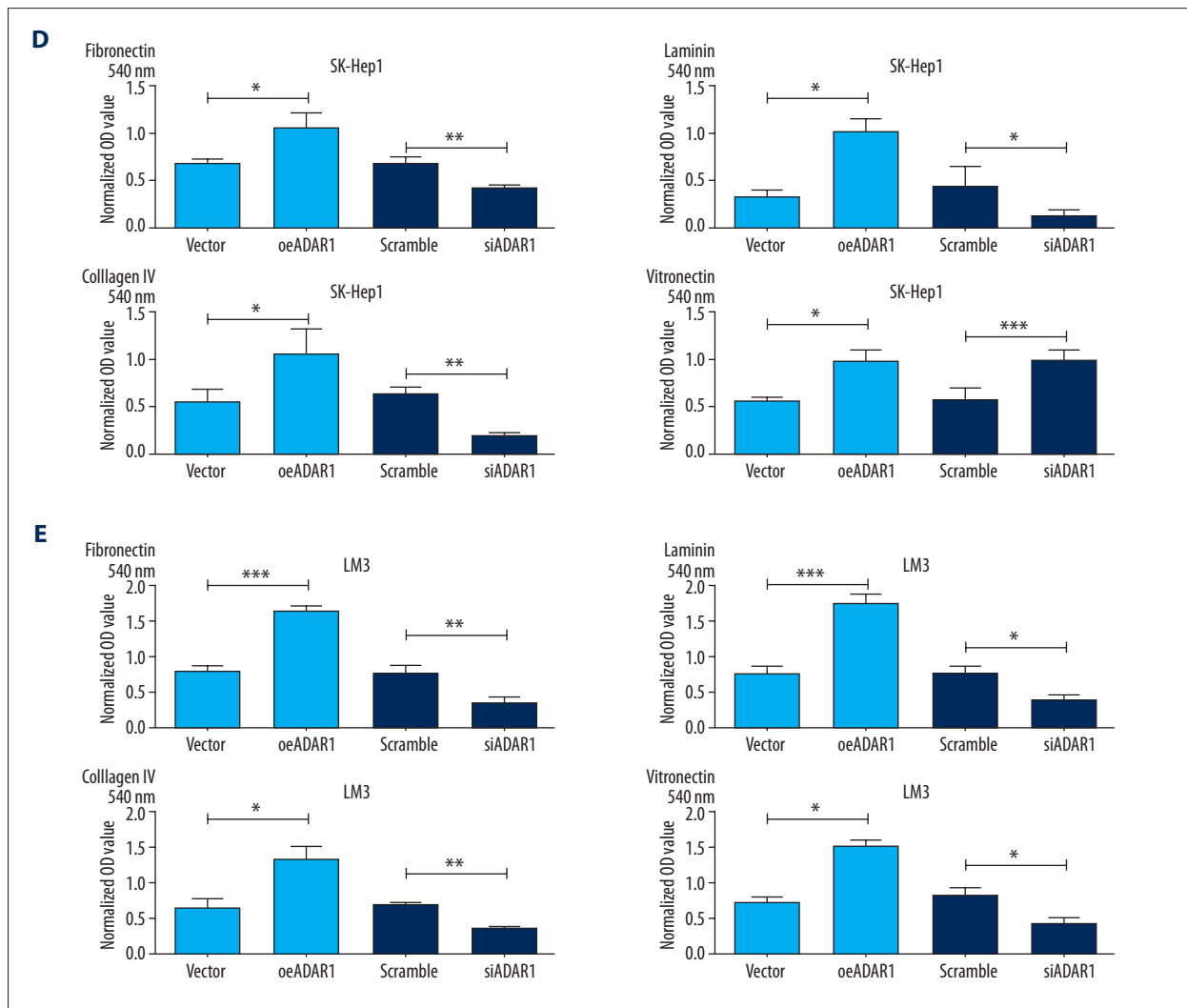
Figure 2. ADAR1 p110 could promote HCC cells to attach to ECM elements. (**A**) We applied a lentivirus system to modify the expression level of ADAR1 p110 in HCC cells. The overexpression or knock-down results were verified by immunoblots. (**B, C**) We performed static cell adhesion assays in SK-Hep1 and LM3 cells. The ADAR1 p110 of these tumor cells were modulated before. Briefly, the culture plates were pre-coated with matrigel and then HCC cells were seeded for a certain period (30 min for SK-Hep1 and 4 h for LM3). After that, the cells were washed by PBS and stained to observe the attached tumor cells. (**D, E**) We used SK-Hep1 and LM3 to test the adhering ability of cells to various components of ECM. Briefly, the cells with different levels of ADAR1 p110 were subjected to fibronectin-, laminin-, collagen IV-, and vitronectin-coated wells and allowed to adhere for certain periods of time (SK-Hep1 for 30 min and LM3 for 120 min). The cells were then stained with crystal violet and read in a colormetric reader (540 nm). All of the results are from at least 3 independent experiments. Data shown are mean ±SD. *** P<0.001, ** P<0.01, *P<0.05.

Fluorescence microscopy

For cell morphology study, we seeded target cells on chamber glass culture slides and fixed the cells with 4% paraformalde-hyde. Then, ActinRed 555 ReadyProbes Reagent (Thermo Fisher) was used for cell F-actin staining according to the manufacturer's instruction. The nuclei were detected by DAPI (Sigma-Aldrich; 10 nM). After staining, cells were then sealed with cover slides and observed by using a confocal microscope.

Patient selection and Immunohistochemistry (IHC) staining

We randomly selected 23 patients' HCC samples for study. Theses patients' samples were collected at our hospital starting from 1 September 2014 to 1 October 2016. This project was approved by the Research Ethics Committee of our hospital. The pathology records were reviewed to identify samples with confirmed hepatocellular carcinoma. The IHC slide scoring for ADAR1 were reviewed by 2 pathologists in a double-blind manner. The staining results were measured semiquantitatively
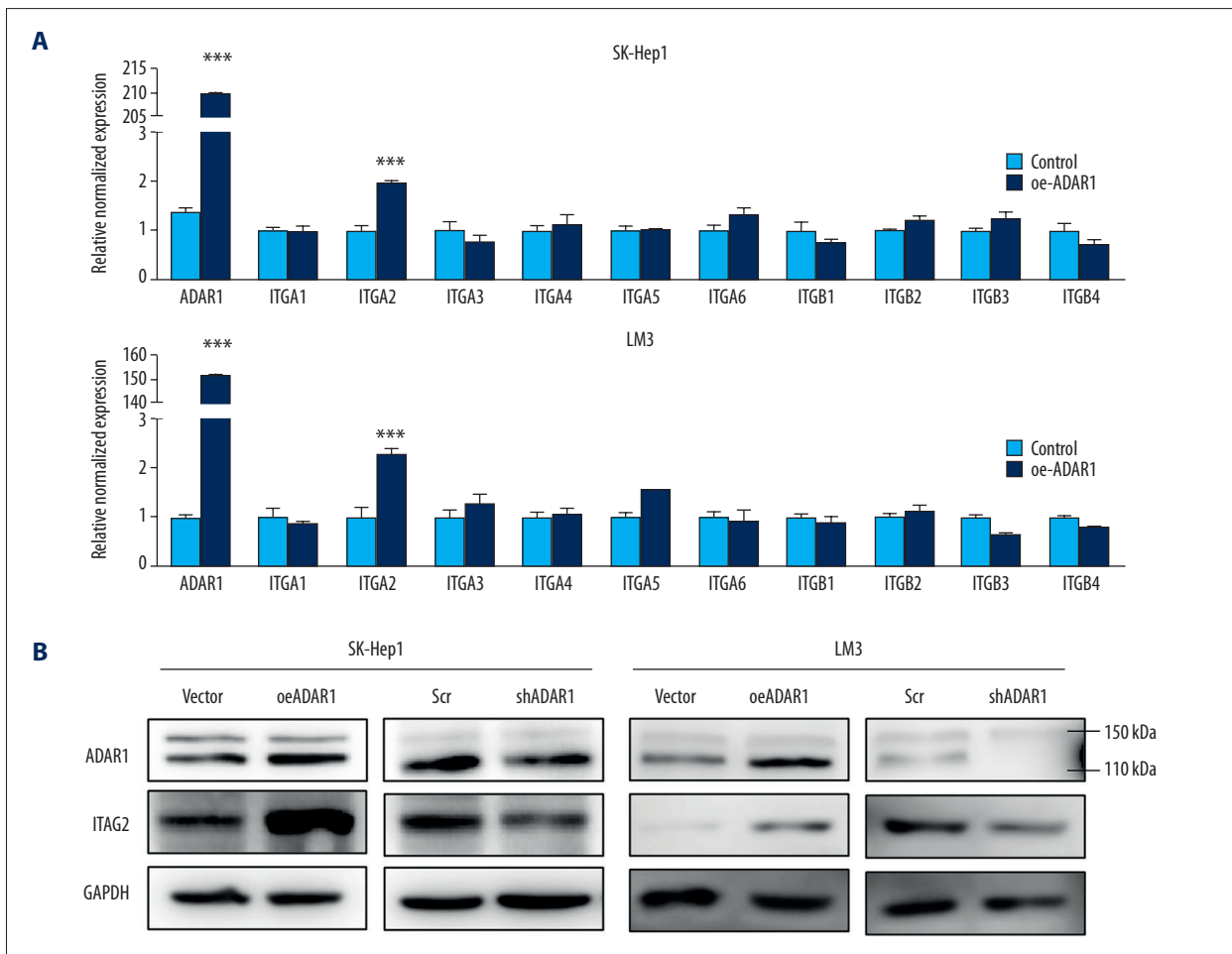
Figure 3. ADAR1 p110 could increase ITGA2 expression both at mRNA level and protein level. (**A**) We extracted total RNA of SK-Hep1 and LM3 that overexpressed ADAR1 p110 and performed RT-qPCR to screen a panel of integrin members. Among the detected integrins, ITGA2 mRNA seemed to be most changed. (**B**) To verify the RT-qPCR result, immunoblotting was performed to detect ITGA2 in SK-Hep1 and LM3 cells that modified ADAR1 p110. ITGA2-specific antibody was used in this assay. Data shown are mean ±SD. *** P<0.001, ** P<0.01, * P<0.05.

and classified into 4 levels: (–), (+), (++), and (+++). The criteria for classification was set as follows: (–), there is less than 50% staining of nuclear ADAR1 in any of the tumor cells/field; (+), there is nuclear ADAR1 staining in 50% to 70% of the tumor cells with any intensity; (++), there is staining in more than 70% of the tumor cells with moderate intensity of nuclear ADAR1; and (+++), there is staining in more than 70% of the tumor cells nuclei with strong intensity of ADAR1. Representative examples of IHC staining for ADAR1 are shown in Figure 1.

IHC stains for ADAR1 were performed using the standard streptavidin-biotin-peroxidase immunostaining procedure. The antibody used for anti-ADAR1 was a poly-cloned one purchased from Abcam (ab88574) with the concentration diluted to 1: 100. Appropriate positive and negative controls were used in each case.

Nude mouse liver orthotopic tumor model

Ten male 6- to 8-week-old nude mice were used and randomly divided into 2 groups. The cells were resuspended in PBS with ECM Matrigel at a 1: 1 ratio. Each group was injected with vector or oeADAR p110 cells into the left lobe of the liver. After injection, the injection spot was held by a cotton bud for 3 min to prevent bleeding. Next, the abdominal cavity was closed by suture.

The mice were sacrificed after 8 weeks, and the liver tumors were isolated for weighing. Before weighing, the isolated tumors were maintained in PBS. The lung was also isolated for metastasis evaluation. All animal studies were approved by the Ethics Committee of Experimental Animals of Zhejiang University and performed under the supervision and guidelines of the Zhejiang University.
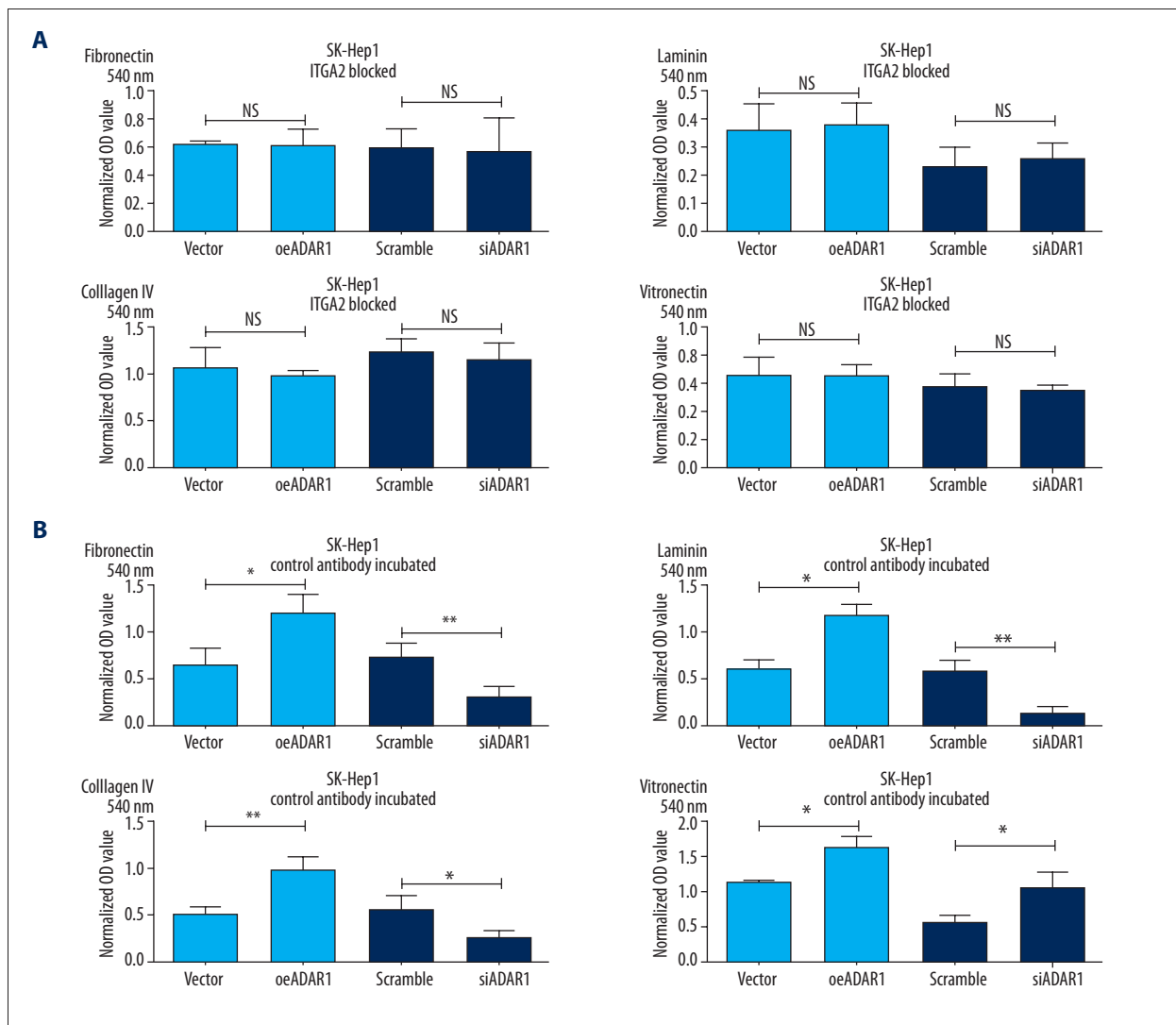
Statistical analysis

The data are expressed as the means ±SD from at least 3 independent experiments. Statistical analyses we applied in this study includes the unpaired *t* test, one-way ANOVA, and Spearman's correlation. The analyses were performed with SPSS 17.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.
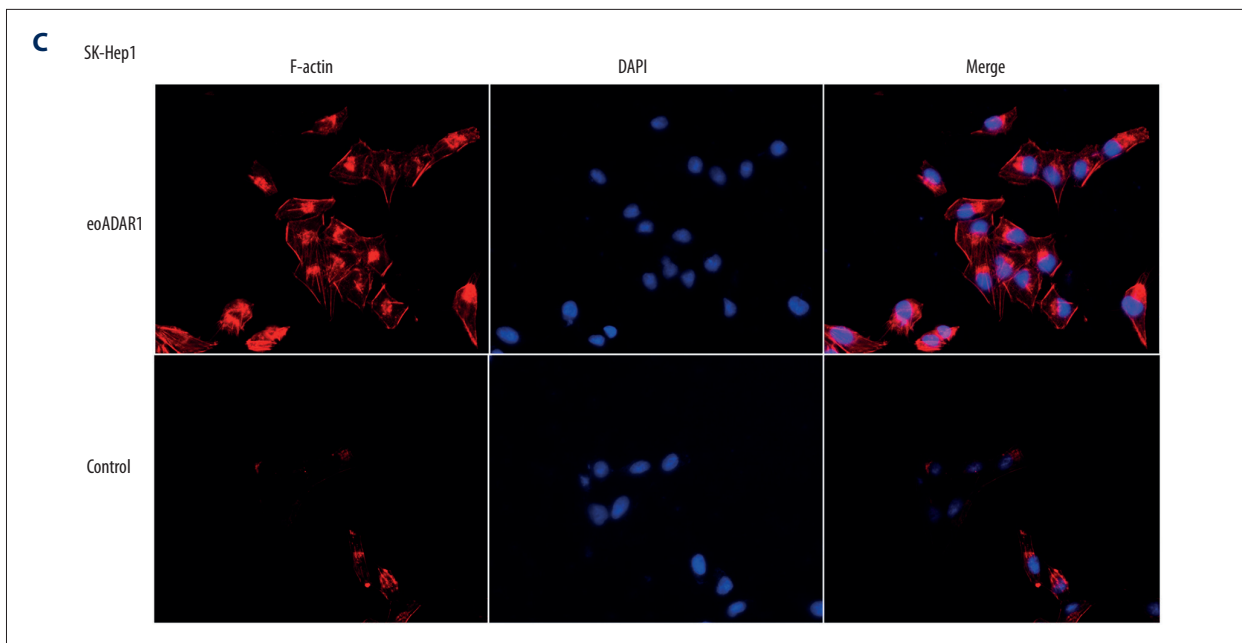
Results

ADAR1 p110 enhances HCC cell adhesion to extracellular matrix

Previous reports have indicated a tumor-promoter role of ADAR1 in a variety of malignancies, including hepatocellular carcinoma [22,23]. We tested whether ADAR1 p110, an isoform driven by a constitutively active promoter and expressed higher than IFN induced p150 in HCC, has an effect on tumor adhesion behavior, since there is no previous evidence that ADAR1 can influence this aspect of malignancy. First, we established ADAR1 p110 overexpression and knocked down cell lines with lentivirus (Figure 2A). Next, we performed adhesion assays to detect HCC tumor cells dynamic adhesion function. Briefly, cells with different statuses of ADAR1 p110 were seeded in matrigel-coated plates and stained later (30 min for SK-Hep1 and 4 h for LM3). The results showed ADAR1 p110 dramatically enhanced tumor cell adhesion to ECM (Figure 2B, 2C). When we coated the plates with various elements of ECM, we found ADAR1 p110 made HCC cells adhere to fibronectin, laminin, collagen IV, and vitronectin tighter and faster (Figure 2D, 2E). These results together suggest that ADAR1 p110 affects binding between HCC tumor cells and ECM substrates.

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Figure 4. ADAR1 p110 enhanced HCC adhesion ability through up-regulating ITGA2. (**A**) We blocked ITGA2 of SK-Hep1 cells by adding anti-integrin α2 antibody into cell culture medium. After finishing blocking, cell adhesion assay was performed according to the same procedure. OD values were measured and compared between groups with different ADAR1 p110 expression. (**B**) We used anti-GAPDH as control antibody for blocking assay. A similar procedure was performed to see if the control antibody would not influence the adhesion difference among groups. (**C**) In order to observe stress fibers in cells with overexpressed ADAR1 p110, we stained F-actin in SK-Hep1 cells. Briefly, cells were seeded on glass culture slides and then fixed with 4% paraformaldehyde. F-actin and nuclei were stained by rhodamine phalloidin and DAPI, respectively. Cells were then sealed with cover slides and observed under a confocal microscope. Data shown are mean±SD. NS – non-significant. *** P<0.001, ** P<0.01, * P<0.05.

ADAR1 p110 up-regulates ITGA2 expression both at mRNA level and protein level

Many studies have elaborated the role of integrin in tumors and showed this family is especially crucial to the metastasis of solid tumors [9]. To find the possible reason for phenomenon we observed, we screened a panel of integrins that may be linked to tumor biological behavior by RT-qPCR. The result suggest integrin α2 might be regulated by ADAR1 p110 (Figure 3A). Next, we verified this finding in SK-Hep1 and LM3 cells by immunoblots and reached a consistent result (Figure 3B).

ADAR1 p110 increases tumor adhesion via boosting ITGA2

To verify that the enhanced tumor cell adhesion is due to ADAR1 p110-induced ITGA2, we used ITGA2 antibody to block our HCC cells before performing cell adhesion assays. The results showed the adhesion among cells expressing different ADAR1 p110 become similar after ITGA2 is blocked (Figure 4A), but when using control antibody, (GAPDH) the differences remained (Figure 4B). We also observed that overexpressing ADAR1 p110 resulted in more actin stress fibers in SK-Hep1 cells compared to the control group cells (Figure 4B), suggesting ADAR1 participates in cellular focal adhesion signaling.

ITGA2 expression is positively correlated with ADAR1 in human HCC samples

Since we have showed a connection between ADAR1 and ITGA2 *in vitro*, the possible correlation of these 2 molecules in clinical settings attracted our attention. To verify our hypothesis, we collected 23 samples of human HCC from our hospital from 1 September 2014 to 1 October 2016. Next, we performed IHC staining for ADAR1 and classified into 4 groups based on ADAR1 expression level. Typical images for –, +, ++ and +++ are shown (Figure 1A). We extracted total RNA from these HCC samples and assayed ITGA2 expression by RT-qPCR (Figure 1B). The results were divided into 3 groups based on their expression grades: low (relative normalized expression 0–0.6), medium (0.6–2.0), and high (above 2.0). After that, Spearman correlation testing was performed and a positive correlation (R=0.5214) was identified (Figure 1C). These data generated from clinical samples revealed ADAR1 can regulate ITGA2 not only in cell line experiments.

ADAR1 promoted tumor metastasis in the orthotopic HCC mouse model

To verify all the above *in vitro* cell lines data *in vivo*, we developed an orthotopic xenograft nude mouse model, as previous
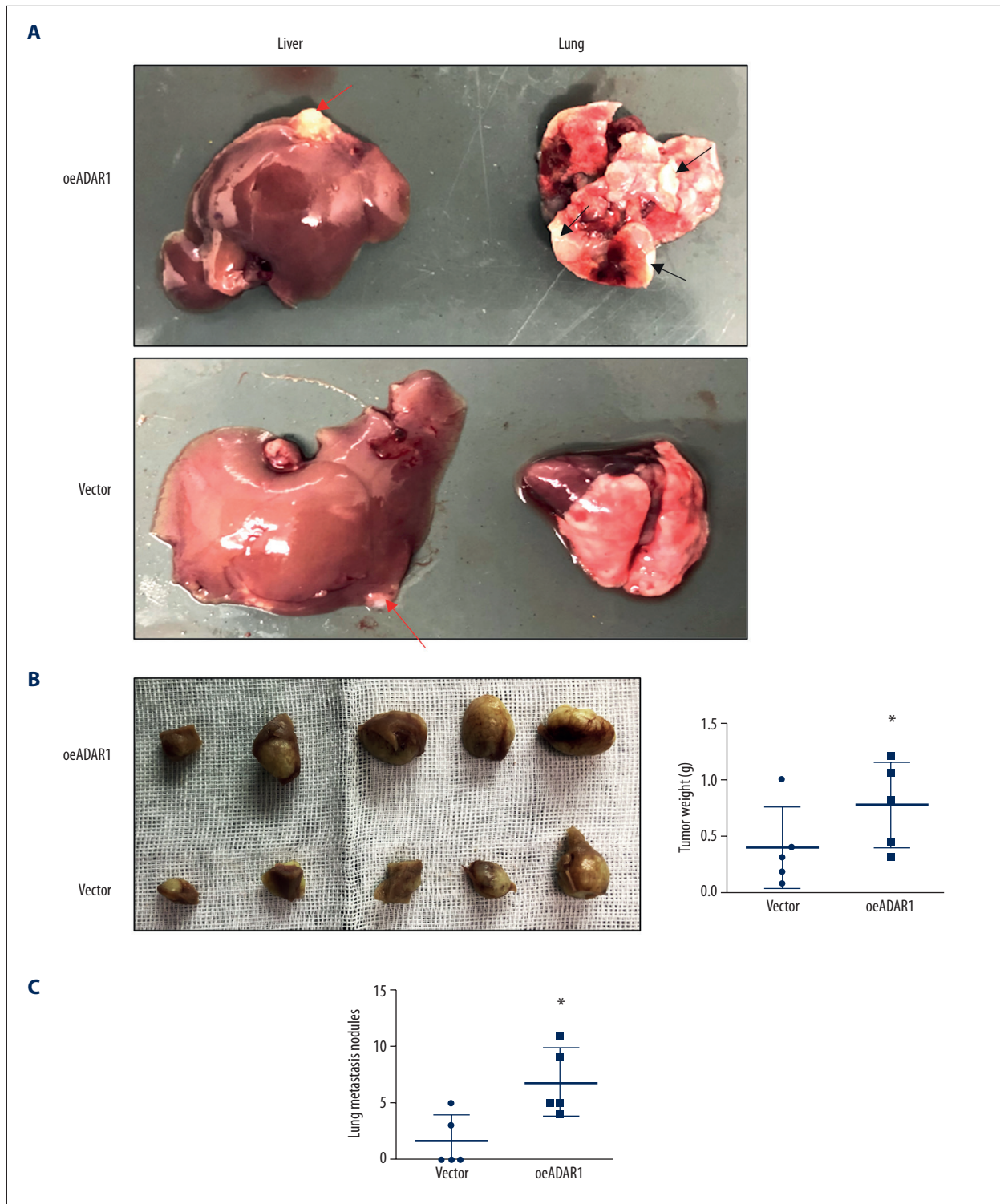
Figure 5. Orthotopic HCC mice model verified that ADAR1 could promote tumor metastasis. (**A**) We isolated liver and lungs after sacrificing the mouse to observe the orthotopic tumor (red arrows) and metastasis foci (black arrows). (**B**) Isolated tumors from liver were kept in PBS before weighing (**left panel**). All the orthotopic tumors were carefully weighed and compared (**right panel**). (**C**) We counted all the metastasis nodules from isolated lungs. The metastasis nodule was defined as abnormal mass which could be located with naked eyes in isolated lungs. Data shown are mean ±SD. *** P<0.001, ** P<0.01, * P<0.05.

described [23]. Briefly, HCC cells SK-Hep1 w/wo overexpressing ADAR1 p110 were injected into left lobes of nude mouse livers. Eight weeks after injection, the mice were sacrificed and the tumors were isolated for weighing. Also, the lungs were evaluated for metastasis foci counting (Figure 5A). The tumor weights showed that ADAR1 significantly promoted tumor growth in our mouse model (Figure 5B). Meanwhile, the group overexpressing ADAR1 showed higher lung metastasis rates by counting the metastasis nodules (Figure 5C). These results strongly support our hypothesis, since the previous study did not mention the metastasis observation in their mouse model [22].

Discussion

In this study, we elaborated how ADAR1 p110 promotes tumor metastasis in liver cancer, which adds information missing from the previous report that ADAR1 works as an oncogene via causing unbalanced RNA editing [22]. This function is achieved by up-regulating ITGA2 expression both at RNA and protein level according to our data. After ITGA2 was boosted by ADAR1 p110, HCC tumor cells tended to interact with ECM components faster and tighter. This phenotype was further verified by neutralizing antibody assay and stress fiber staining. Data generated from clinical samples and orthotopic mouse models also confirmed our hypothesis. Unfortunately, our results in the mouse model could not represent the intrahepatic metastasis status due to technical issues. We believe that live imaging of small animals could detect the small intrahepatic metastasis and solve this problem in future research.

Recently, several groups have reported a cancer promoter role of ADAR1 in HCC. One claimed unbalanced RNA editing induced by ADAR1 overexpression influenced HCC biological behavior [22]. Although both ADAR1 p110 and p150 were proved to be functional in HCC, several publications indicated p110 was expressed much more in HCC, which is consistent with our data [22,23]. Similarly, another group found androgen receptor-induced ADAR1 p110 could impair the circular RNA expression panel in liver cancer and this phenomenon may contribute to the sex ratio disparity in HCC [23]. However, their studies did not link ADAR1 to molecules that directly relate to metastasis, which means our findings could be a supplement to fully illuminate how ADAR1 promotes tumor invasion. As for the mechanism by which ADAR1 regulates ITGA2 in our settings, we assumed it went through an indirect pathway and influenced certain transcription factors that target ITGA2 due to both mRNA and protein changes. Previous reports mainly explained oncogenic role of ADAR1 from its RNA editing aspect. In brief, a whole panel of unbalanced RNA editing induced by

abnormal ADAR1 made HCC progress. In our study, we found ITGA2, possibly due to RNA editing or other possible indirect functions of ADAR1. For Filamin B and AZIN1, ADAR1 mainly influences their RNA editing process and increases the A to I rate [22]. However, we noticed RNA expression change of ITGA2 due to ADAR1 in our study, and this phenotype could be induced by other mechanism of ADAR1 [23,27].

Although the role of integrins in cancer has been well documented, the functions of some members of the family remain controversial in certain circumstances. For example, ITGA2 was reported to be overexpressed in squamous cell carcinoma and might serve as a tumor promoter [28], while the loss of ITGA2 in breast cancer could be a biomarker for tumor progression [29]. When we neutralized ITGA2 and performed adhesion assay in our study, the results suggested ITGA2 plays an unwanted role. Based on these results, it appears that ITGA2 is mainly an oncogene in HCC [21]. However, the effects of various combinations with integrin β still further study.

Interestingly, researchers found integrin might be able to participate in immune surveillance for tumor via a phenomenon called "integrin trans-regulation", which could provide a novel way to inspect integrin during tumor progression [30]. On the other hand, Done et al. reported ITGA2, but not in its combination form with integrin β, could help cancer spread to distant organs via the HMGA2-FOXL2-ITGA2 pathway [31]. All these findings suggested us that the subunit of integrin might exert some unrealized functions in tumors rather than the classic heterodimer, and this requires further investigation.

Conclusions

Our findings indicate a novel regulation between ADAR1 p110 and ITGA2 in hepatocellular carcinoma, showing that overexpressed ADAR1 p110 in tumors can increase ITGA2 as well. This phenotype directly enhanced adhesion ability of tumor cells, which further initiated cancer cell invasion. These results were verified by our clinical samples and orthotopic mouse model. As we mentioned in the introduction section, we could develop a better approach to prevent early-stage HCC progression as more information is reported on the mechanisms of HCC metastasis, which is also a top priority for researchers involved in cancer research.

Conflicts of interest

None.

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