

A-kinase anchoring protein 12 is downregulated in human hepatocellular carcinoma and its deficiency in mice aggravates thioacetamide-induced liver injury

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Received March 9, 2018; Accepted July 13, 2018

DOI: 10.3892/ol.2018.9396

Abstract. AKAP12 belongs to A-kinase anchoring protein (AKAP) family of scaffold proteins and is known as a tumor suppressor in several human cancer types. Its role as a tumor suppressor in hepatocellular carcinoma (HCC) was proposed due to its downregulation and epigenetic modification in human HCC; however, the effect of its deficiency on liver injuries, such as liver fibrosis and cancer has been poorly studied. By analyzing tumor and non-tumor tissues of 15 patients with HCC, it was confirmed that AKAP12 expression was downregulated in human HCC as compared with adjacent non-tumor tissues. Immunohistochemical staining of mouse liver tissue for AKAP12 revealed that its sinusoidal expression was diminished in capillarized endothelium after

8 weeks of thioacetamide (TAA) administration. AKAP12 deficiency resulted in the promotion of ductular response of biliary epithelial cells, whereas overall fibrosis and myofibroblast activation were comparable between genotypes after short-term TAA treatment. The mRNA expressions of some fibrosis-related genes such as those encoding epithelial cell adhesion molecule, collagen type 1 α 1 and elastin were upregulated in liver tissues of AKAP12-knockout mice. Long-term administration of TAA for 26 weeks led to the development of liver tumors; the incidence of tumor development was higher in AKAP12-deficient mice than in wild-type littermates. Together, these results suggest that AKAP12 functions as a tumor suppressor in liver cancer and is associated with the regulation of hepatic non-parenchymal cells.

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Abbreviations: AKAP, A-kinase anchoring protein; HCC, hepatocellular carcinoma; TAA, thioacetamide; ECM, extracellular matrix; WT, wild-type; KO, knockout; qPCR, quantitative polymerase chain reaction; HBV, hepatitis B virus; EpCAM, epithelial cell adhesion molecule

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Key words: liver fibrosis, hepatocellular carcinoma, thioacetamide, A-kinase anchoring protein 12, liver sinusoid

Introduction

Chronic liver diseases such as cirrhosis and liver cancers are one of the leading causes of death worldwide. The development of chronic liver diseases is associated with a wide range of liver injuries, including virus infection, alcohol consumption, and metabolic disorders (1,2). Liver cancer is known to mostly develop under fibrotic background (3) and hepatic non-parenchymal cells play central roles in the progression of liver fibrosis (2,4). Therefore, the progression from liver fibrosis to liver cancer is thought to be coordinately regulated through the oncogenic activation of hepatocytes and microenvironmental modulation of non-parenchymal cells.

A-kinase anchoring proteins (AKAPs) spatio-temporally regulate cellular signalings by scaffolding effector proteins (5,6). AKAP12 (also called as gravin/SSeCKS/AKAP250) interacts with signaling mediators such as protein kinase A, protein

kinase C, and Src, and modulates a variety of cellular and physiological events, including cytoskeletal remodeling, cell migration, blood-brain barrier development, and oncogenic processes (7,8). Furthermore, AKAP12 is recognized as a tumor suppressor, as evident from the downregulation of its expression in several human cancers such as prostate, ovarian, gastric, and breast cancers. AKAP12 downregulation is often caused by chromosomal hypermethylation or deletion (9-12). Furthermore, its overexpression in cells is known to inhibit oncogenic and metastatic properties, whereas its deficiency enhances the susceptibility to oncogenic transformation (13,14).

Previous studies have reported the downregulation of AKAP12 in human hepatocellular carcinoma (HCC) and its association with promoter hypermethylation, chromosomal deletion, and some specific microRNAs (15-17). Forced overexpression or silencing of AKAP12 gene in HCC cell lines revealed that AKAP12 inhibits HCC cell growth and survival, suggestive of its tumor suppressor activity (17). However, the role of AKAP12 at the level of liver organ as well as its functions in hepatic non-parenchymal cells remain to be investigated.

In the present study, we analyzed the expression pattern of AKAP12 in normal and injured liver tissue and compared thioacetamide (TAA)-induced liver injuries between wild-type (WT) and AKAP12-knockout (KO) mice to evaluate the role of AKAP12 in the progression of liver fibrosis and cancer.

Materials and methods

Animals and TAA-induced liver injuries. All mouse experiments were reviewed and approved by the Committee for Care and Use of Laboratory Animals at Seoul National University and performed according to the Guide for Animal Experiments edited by the Korean Academy for Medical Sciences. WT and AKAP12-deficient (AKAP12 KO) C57BL/6 mice were bred and maintained as previously described (10). Hepatic fibrosis and tumorigenesis were induced by TAA in weight-matched 8- to 10-week-old WT or AKAP12-KO male mice. For fibrosis model, TAA was intraperitoneally injected thrice a week at a dose of 150 mg/kg for 8 weeks (18,19). The vehicle group received saline thrice a week for 8 weeks. To generate liver tumor, mice were fed with drinking water containing TAA at 300 mg/l for 26 weeks (20). At the end of the experimental period, mice were euthanized via deep anesthesia and cardiac perfusion.

Humantissue specimens. HCC tissues and adjacent non-tumorous liver samples were obtained from patients who received surgical resection for HCC at Asan Medical Center, Seoul, Korea. Informed consent was obtained from all patients. HCC patients who meet the following criteria are included for the analysis: at least 20 years of age and surgical specimen is histologically confirmed as HCC. Patients who accompany other malignancy or Child-Pugh class C disease are excluded. To determine HBV infection, HBsAg were detected using microparticle enzyme immunoassay (Abbott Laboratories, Chicago, IL, USA) or immunoradiometric assay kit (DiaSorin S.p.A., Vercelli, Italy). Patients' clinical characteristics are summarized in Table I. The study was approved by the Institutional Review Board of the Asan Medical Center, Seoul, Korea.

Histological analysis and immunohistochemistry. To visualize the deposition of extracellular matrix (ECM) proteins, paraffin sections were de-paraffinized and stained with picro-sirius red (Abcam, Cambridge, MA, USA) according to manufacturer's instructions. Immunohistochemical staining was performed on paraffin or frozen liver sections. To stain the paraffin sections, antigen retrieval was performed for 30 min at 95°C in Tris buffer (pH 9.0). After blocking with 5% normal donkey serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in phosphate-buffered saline (PBS), the sections were overnight incubated at 4°C with primary antibodies for AKAP12 (I. Gelman, Roswell Park Cancer Institute), α SMA (Agilent Technologies, Inc., Santa Clara, CA, USA), laminin (Sigma-Aldrich; Merck KGaA), SE-1 (R&D Systems, Inc., Minneapolis, MN, USA), and epithelial cell adhesion molecule (EpCAM; BD Biosciences, Franklin Lakes, NJ, USA). After extensive washing with PBS containing 0.1% Tween-20 solution, the sections were treated with Alexa 488 or 546-conjugated secondary antibodies (1:750; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at room temperature, followed by counter staining with Hoechst stain (Sigma-Aldrich; Merck KGaA). Fluorescent images were taken under a confocal microscope (Carl Zeiss AG, Oberkochen, Germany) and immuno-positive areas were quantified by ImageJ software (21). A total of 5-10 low magnification (x50) images from a single animal were quantified and the mean value was considered as a representative value for that animal.

Isolation of RNA and quantitative polymerase chain reaction (qPCR) analysis. Liver tissues were homogenized using TissueLyser II (Qiagen, Hilden, Germany) in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and total RNA was extracted according to the manufacturer's instructions. Two micrograms of RNA from each sample was reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega Corporation, Madison, WI, USA). Quantitative real-time PCR was performed using StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with RealHelix qPCR kit (NanoHelix Co., Ltd., Seoul, Korea). The relative mRNA levels were normalized by a housekeeping gene encoding glyceraldehyde-3-phosphate (*GAPDH*). Primer sequences used in this study are provided in Table II.

Western blot analysis. Liver tissues were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 0.5% NP-40, phosphatase inhibitor cocktail (Sigma) and proteinase inhibitor cocktail (EMD Millipore, Billerica, MA, USA). Protein concentrations were determined using a bicinchoninic acid (BCA) Assay kit (Thermo Fisher Scientific, Inc.). 20 μ g of lysates were resolved on polyacrylamide gel and then immunoblotted for AKAP12 (I. Gelman, Roswell Park Cancer Institute) and vinculin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as described previously (22).

Statistical analysis. All data are expressed as mean \pm standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Tukey's tests or two-tailed

Table I. Clinical characteristics of human subjects included in the present study.

Patient no.	Age	Sex	Etiology	Max. tumor size (cm)	Edmondson-Steiner grade	Cirrhosis
1	69	M	HBV	7.1	3	No
2	42	M	HBV	1.8	3	No
3	48	M	HBV	3.5	2	Yes
4	78	F	HBV	5.0	4	Yes
5	43	M	HBV	3.8	4	No
6	70	F	HBV	5.3	3	Yes
7	56	M	HBV	2.2	3	No
8	55	F	HBV	11.8	3	Yes
9	51	M	HBV	1.9	3	Yes
10	59	M	HBV	3.2	2	Yes
11	57	F	HBV	3.0	4	No
12	58	M	HBV	2.3	3	No
13	74	M	NBNC	10.5	4	No
14	45	M	HCV	2.5	3	No
15	71	M	Alcohol	1.4	4	No

M, male; F, female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-HBV and non-HCV.

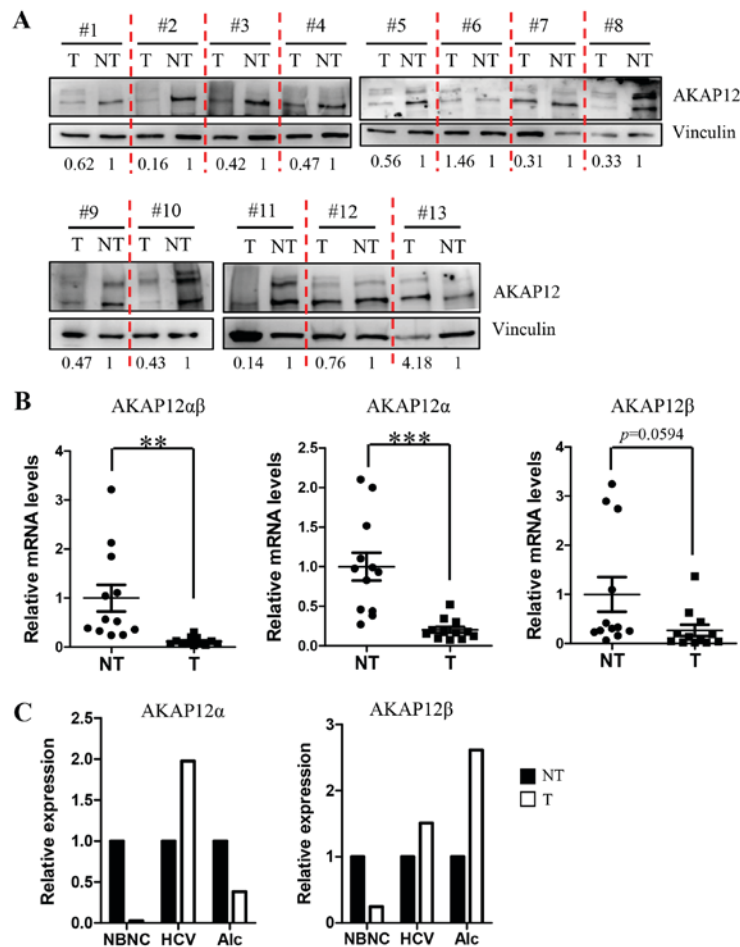


Figure 1. AKAP12 is downregulated in human HCC. (A) Western blots were obtained from tissue lysates isolated from HCC tumors (T) and adjacent non-tumor tissues (NT). Vinculin was used as a loading control. Relative AKAP12 band intensities of T/NT pairs are shown under western blot images after normalization with vinculin intensities. (B) Transcript levels of total AKAP12 (left), AKAP12α isoform (middle), and AKAP12β isoform (right) were compared between 13 tumor/non-tumor tissues carrying HBV infection using qRT-PCR analysis. Data are expressed as mean ± SEM. **P<0.01, ***P<0.001, n=13. (C) Transcript levels of AKAP12α (left) and AKAP12β (right) isoforms were compared between one each of non-HBV/non-HCV (NBNC)-, HCV-, and alcohol (Alc)-associated HCC tissue. Graphs are relative level of mRNAs to non-tumor tissues. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non-HBV and non-HCV; qRT-PCR, quantitative real-time polymerase chain reaction; mean ± standard error of the mean (SEM).

Student's t-test was used for statistical analyses. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism v.5.00 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

AKAP12 is downregulated in human HCC tissues positive for hepatitis B virus (HBV) infection. Previous reports from three independent groups revealed the significant downregulation in AKAP12 expression in human HCC samples (15-17). To confirm the reduced AKAP12 gene expression in Korean HCC patients, we analyzed protein lysates from 13 pairs of HCC and adjacent non-tumor tissues by western blotting. Of these, 11 HCC samples exhibited reduced level of AKAP12 protein as compared with the adjacent non-tumor tissues (Fig. 1A). The remaining two HCC samples showed an increase in the expression of AKAP12 levels and were negative for HBV infection. On the other hand, the 11 samples with reduced AKAP12 expression in the tumor were positive for HBV infection. We analyzed transcript levels of AKAP12 isoforms alpha and beta separately in 12 HBV-associated HCC tissues and one each of non-HBV/non-HCV (NBNC)-, alcohol- and HCV-associated HCC tissues (Fig. 1B and C). Transcript levels of total AKAP12 $\alpha\beta$ amplified with common AKAP12 $\alpha\beta$ primers showed a significant reduction in AKAP12 expression in HCC tumors as compared to adjacent non-tumor tissues of HBV groups. We performed qPCR analysis to detect the expression of AKAP12 α and AKAP12 β isoforms using isoform-specific primer sets and found less significant reduction in AKAP12 β gene expression in tumor tissues ($P=0.0594$), whereas the reduction in the expression of AKAP12 α gene in tumors was highly significant ($P=0.0002$) (Fig. 1B). In contrast, HCC samples with other etiologies showed variable results (Fig. 1C), although we failed to analyze statistical differences by etiologies owing to the limited number of samples.

Sinusoidal AKAP12 expression is reduced in fibrotic regions following TAA administration for 8 weeks. To evaluate the role of AKAP12 in liver fibrosis, we used TAA-induced liver fibrosis model. Picro-sirius red staining revealed that 8 weeks of intraperitoneal TAA injection resulted in the accumulation of ECM from central veins (Fig. 2A). In the vehicle-treated mouse livers, AKAP12 immunoreactivity was observed in the sinusoidal endothelial cells (LSECs) as revealed by co-localization with a LSEC marker, SE-1, whereas hepatocytes were almost negative for AKAP12 staining (Fig. 2B and C). After TAA injection, the sinusoidal AKAP12 expression was reduced in fibrosis regions where an obvious activation of myofibroblasts was detected (Fig. 2C). Contrary to AKAP12 expression pattern in normal and fibrotic livers, laminin expression was nearly absent in normal liver but increased in fibrotic livers, especially in the area of myofibroblast activation (Fig. 2D). These results indicate that TAA administration results in centrilobular fibrosis that involves capillarization of sinusoidal endothelium and that sinusoidal AKAP12 expression is diminished in capillarized microvessels in the fibrotic area.

Comparison of liver injuries between WT and AKAP12-KO mice after short-term TAA injection. We evaluated if

Table II. Primer sequences for qPCR detection.

A, Human primers	
Gene	Sequences (5'-3')
<i>AKAP12$\alpha\beta$</i>	
Forward	CAGAAGTCAGAGCAAGTGCC
Reverse	ACCTGAGGGGGAACATTTGA
<i>AKAP12α</i>	
Forward	AACGGTCAAGGAGCCCTAAA
Reverse	CATCTTCAGAGTCTCTCTGTCCAA
<i>AKAP12β</i>	
Forward	CCGCTAAGCTGATCTCCTGT
Reverse	CATCTTCAGAGTCTCTCTGTCCAA
<i>GAPDH</i>	
Forward	TGAACGGGAAGCTCACTGG
Reverse	TCCACCACCCTGTTGCTGTA
B, Mouse primers	
Gene	Sequences (5'->3')
<i>coll1a1</i>	
Forward	CATGTTTCAGCTTTGTGGACCT
Reverse	GCAGCTGACTTCAGGGATGT
<i>αSMA</i>	
Forward	GACACCACCCACCCAGAGT
Reverse	ACATAGCTGGAGCAGCGTCT
<i>Elastin</i>	
Forward	GGGCCCTGGTATTGGAGGTC
Reverse	ACTCCACCTCTGGCTCCGTA
<i>EpCAM</i>	
Forward	AGGGGCGATCCAGAACAACG
Reverse	ATGGTCGTAGGGGCTTTCTC
<i>TGF-β1</i>	
Forward	TTGCTTCAGCTCCACAGAGA
Reverse	TGGTTGTAGAGGGCAAGGAC
<i>PDGFa</i>	
Forward	GAGATACCCCGGGAGTTGAT
Reverse	AAATGACCGTCTCTGGTCTTG
<i>PDGFb</i>	
Forward	CCTCGGCCTGTGACTAGAAG
Reverse	GGACGAGGGGAACAACATTA
<i>GAPDH</i>	
Forward	TGAACGGGAAGCTCACTGG
Reverse	TCCACCACCCTGTTGCTGTA

AKAP, A-kinase anchoring protein; gapdh, glyceraldehyde 3-phosphate dehydrogenase; Coll1a1, collagen type I alpha 1; α SMA, alpha smooth muscle actin; EpCAM, epithelial cell adhesion molecule; TGF- β 1, transforming growth factor beta 1; PDGFa, platelet derived growth factor subunit A; PDGFb, platelet derived growth factor subunit B; gapdh, glyceraldehyde 3-phosphate dehydrogenase; qPCR, quantitative polymerase chain reaction.

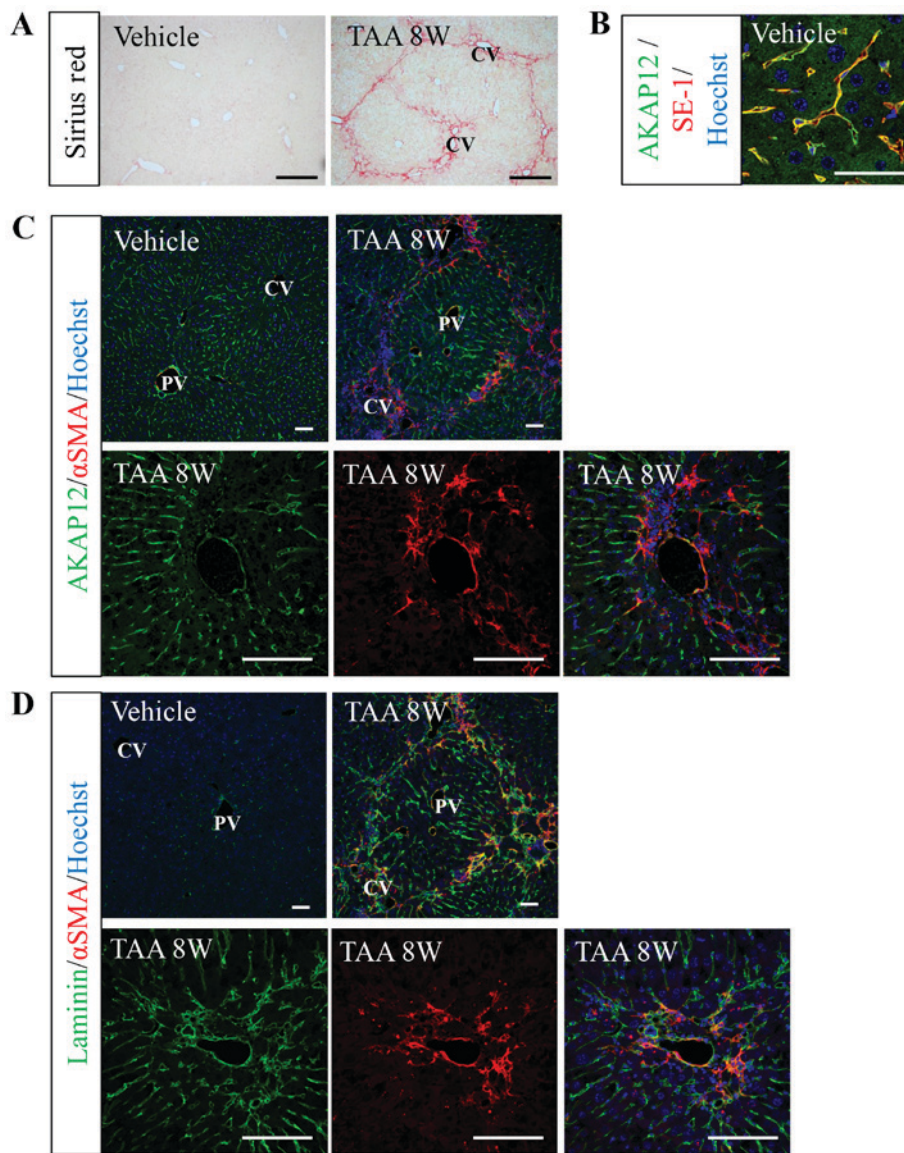


Figure 2. AKAP12 expression is reduced in sinusoids of fibrotic regions after 8 weeks of TAA administration. (A) Mice received intraperitoneal injections of TAA for 8 weeks, as described in Materials and methods. Picro-sirius red staining was performed on liver sections from either vehicle or TAA-injected mice. (B) Double-immunofluorescent staining for AKAP12 (green) and SE-1 (red) showing specific expression of AKAP12 in SE-1-positive LSECs. (C) Double-immunofluorescent staining for AKAP12 (green) and α SMA (red) showing the expression pattern of AKAP12 in control or fibrotic liver. AKAP12 immunoreactivity was prominent along liver sinusoids and portal track in normal liver (upper left), but sinusoids in fibrotic areas were negative for AKAP12 signal. (D) Double-immunofluorescent staining for laminin (green) and α SMA (red) showed that capillarization of liver sinusoids was obvious in fibrotic livers. Scale bars represent 400 μ m in A and 100 μ m in B-D. TAA, thioacetamide; LSECs, liver sinusoidal endothelial cells; CV, central vein; PV, portal vein, α SMA, alpha smooth muscle actin.

AKAP12 deficiency leads to the alteration in liver injury caused by TAA. After 8 weeks of TAA administration, the level of ECM deposition was comparable between livers from AKAP12-KO mice and WT littermates, as revealed by picro-sirius red staining (Fig. 3A). Myofibroblastic activation as well as sinusoidal basement membrane formation were similar between genotypes (Fig. 3B, α SMA and laminin staining). In contrast, EpCAM-positive cholangiocyte expansion was approximately 2.5-fold higher in AKAP12-KO livers than WT controls (Fig. 3B, EpCAM staining). We compared transcript levels of specific fibrosis-related genes in WT and AKAP12-KO livers with qRT-PCR. As evidenced by the results of immunohistochemical staining, EpCAM mRNA level was higher in AKAP12-KO mice than in WT control after 8 weeks of TAA injection

(Fig. 4A, left). Moreover, the transcript levels of certain ECM genes, namely *Colla1* and *elastin*, were significantly increased in AKAP12-KO livers than in WT controls after TAA injection (Fig. 4A, middle and right). However, no significant difference was observed in the level of myofibroblast marker α SMA (Fig. 4B, left) and fibrosis-related soluble factors such as *TGF- β 1*, *PDGFA*, and *PDGFB* (Fig. 4B) between genotypes.

Comparison of hepatic tumorigenesis between WT and AKAP12-KO mice after long-term TAA administration. Long-term TAA administration to rodents is known to generate liver tumors, including hepatocellular adenoma and HCC (20,23,24). As AKAP12 is a potential tumor suppressor in human HCC, we compared the tumorigenesis of WT and

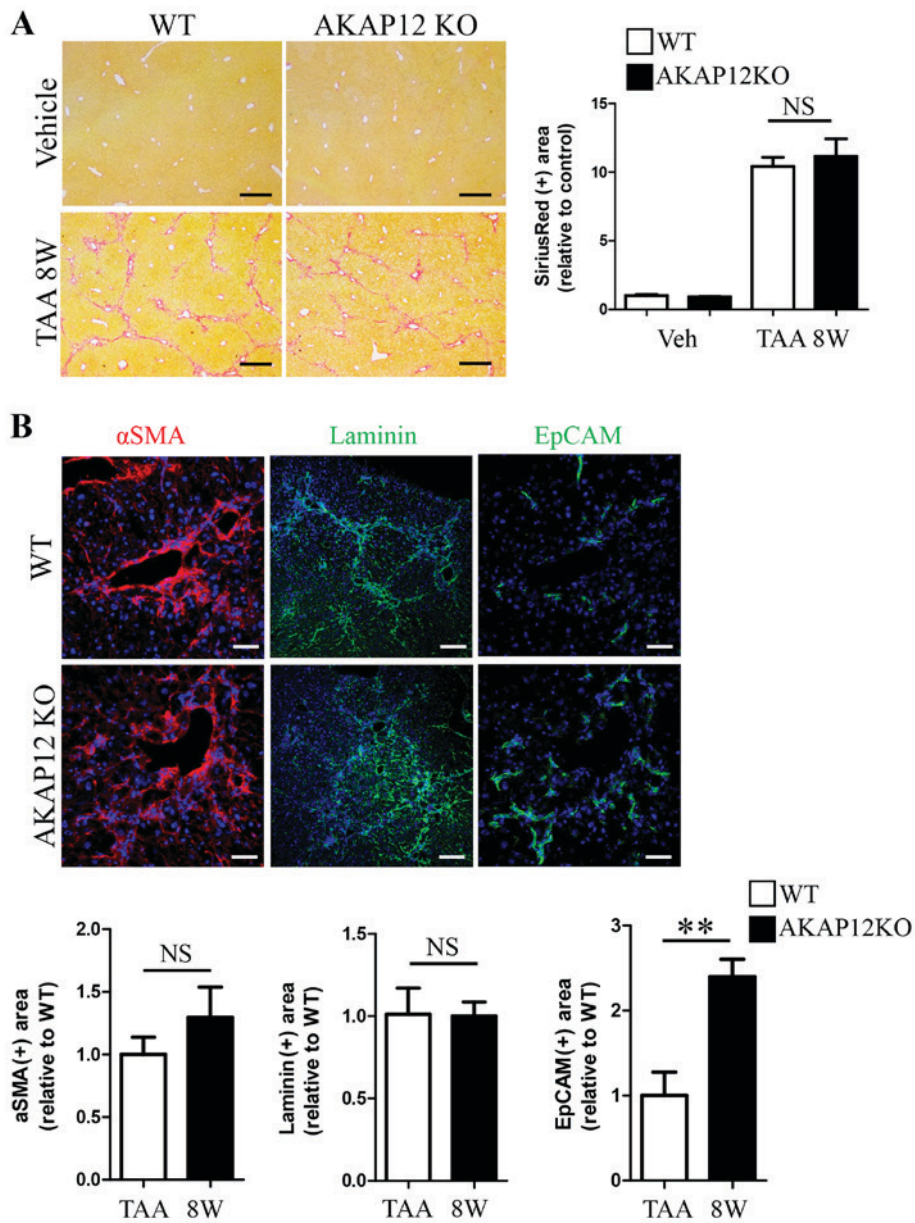


Figure 3. AKAP12-KO mice displayed comparable level of fibrosis after 8 weeks of TAA injection, whereas ductular response was greater in AKAP12 mice than in WT littermates. (A) Overall ECM deposition was detected by picro-sirius red staining after 8 weeks of vehicle or TAA injection. Graph shows quantifications of picro-sirius red-positive area relative to WT control. Data are expressed as mean ± SEM, n=4 in WT veh and AKAP12-KO veh; n=10 in WT TAA 8 W; n=6 in AKAP12-KO TAA 8 W. Scale bars represent 400 μm. (B) Liver sections from WT or AKAP12-KO mice after 8 weeks of TAA injection were subjected to immunofluorescent staining for αSMA (left column), laminin (middle column), and EpCAM (right column). Scale bars indicate 50 μm. Graphs are morphometric quantifications of areas stained by each antibody. Data are expressed as mean ± SEM. n=4 mice per genotype. **P<0.01. AKAP, A-kinase anchoring protein; TAA, thioacetamide; EpCAM, epithelial cell adhesion molecule; NS, not significant; mean ± standard error of the mean (SEM).

AKAP12-KO mice after 26 weeks of TAA administration. Five of nine WT mice developed tumor nodules that were visible to the physical eye and one mouse developed a tumor over 2 mm size with maximum diameter of 7.8 mm (Fig. 5A). In contrast, six of nine AKAP12-KO mice developed tumor nodules visible to the physical eye and three mice developed tumors over 2 mm size with maximum tumor diameter of 7.4/8.3/8.6 mm, respectively (Fig. 5A). Histological analysis with hematoxylin and eosin staining revealed more malignant tumors in AKAP12-KO livers (Fig. 5B). Furthermore, tumors in AKAP12-KO livers were more vascularized than WT tumors, as revealed by laminin staining. Laminin immunoreactivity of adjacent fibrotic area was comparable between

genotypes (Fig. 5C). Therefore, AKAP12 seems to exhibit an inhibitory role in hepatic tumorigenesis and may be associated with the regulation of hepatic non-parenchymal cells such as liver sinusoidal endothelial cells.

Discussion

It is now well-recognized that stromal cells in tumor micro-environment play important roles in tumorigenesis and metastasis (25,26). Tumor-associated microenvironmental cells such as cancer-associated fibroblasts and tumor endothelial cells are profoundly different from their counterparts in normal tissues (25,26) in terms of morphology, behavior, gene

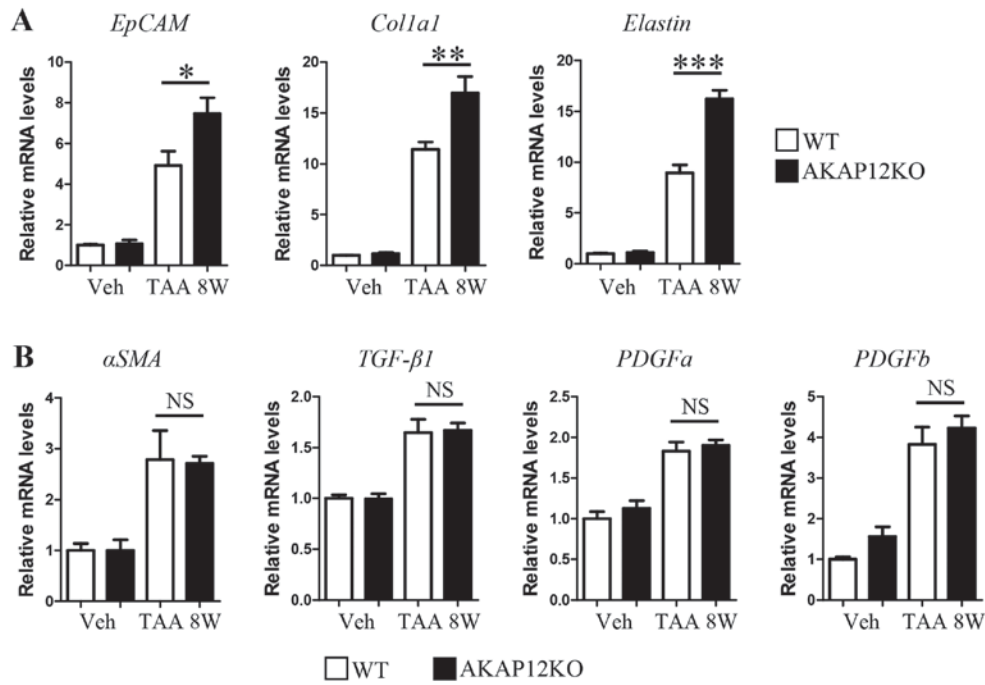


Figure 4. Comparison of fibrosis-related gene expression between WT and AKAP12-KO livers after 8 weeks of TAA administration. (A) Relative mRNA levels of *EpCAM*, *Colla1*, and *elastin* in liver tissues were quantified by qRT-PCR with specific primers. (B) Relative mRNA levels of *α SMA*, *TGF- β 1*, *PDGFa*, and *PDGFb* in liver tissues were quantified by qRT-PCR with specific primers. Data are expressed as mean \pm SEM. n=4 mice per group. *P<0.05; **P<0.01; ***P<0.001; NS, not significant. AKAP, A-kinase anchoring protein; gapdh, glyceraldehyde 3-phosphate dehydrogenase; *Colla1*, collagen type I alpha 1; α SMA, alpha smooth muscle actin; *EpCAM*, epithelial cell adhesion molecule; *TGF- β 1*, transforming growth factor beta 1; *PDGFa*, platelet derived growth factor subunit A; *PDGFb*, platelet derived growth factor subunit B; gapdh, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction.

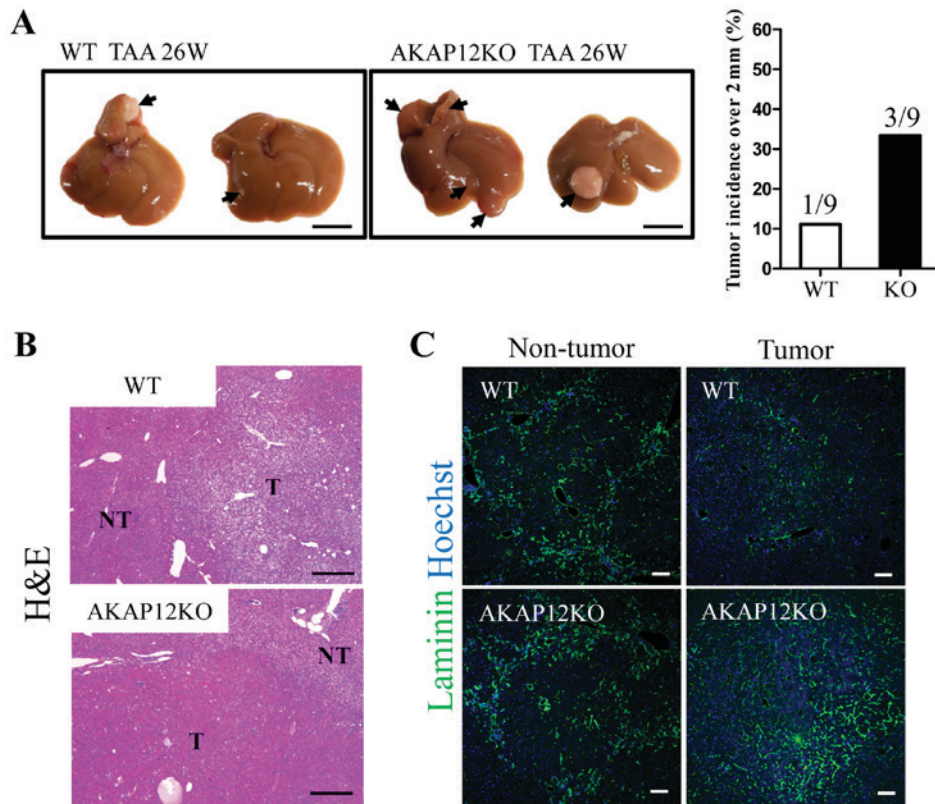


Figure 5. AKAP12-KO mice showed increased incidence of highly vascularized liver tumors after long-term TAA administration. (A) Gross liver images of WT or AKAP12-KO mice after 26 weeks of TAA administration through drinking water. Scale bars indicate 1 cm. Graph shows incidence of liver tumors over 2 mm size. n=9 mice per genotype. (B) Representative H&E images of the liver sections from WT or AKAP12-KO mice after 26 weeks of TAA administration. Scale bars indicate 400 μ m. (C) Immunofluorescent staining for laminin (green) shows capillarized vessels in tumor or non-tumor regions of WT or AKAP12-KO mice livers. Note that tumors developed in AKAP12-KO mice exhibited denser vessels than WT tumors, whereas the level of capillarization in fibrotic non-tumor area was similar between genotypes. Scale bars indicate 100 μ m. AKAP, A-kinase anchoring protein; TAA, thioacetamide; T, tumor region; NT, adjacent non-tumor region.

expressions, and even genetic alterations (27,28). Evidences suggest that some tumor suppressor genes undergo genetic alterations not only in the tumor cells but also in tumor-associated stromal cells. For instance, p53 is often found to be mutated in stromal cells and is associated with tumor grade and metastasis (28-30).

Our immunohistochemistry results reveal the prominent expression of AKAP12 in sinusoidal endothelial cells of normal liver tissue (Fig. 2B), suggestive of the possibility of micro-environmental regulation by AKAP12 in liver injuries. The outcome of short-term and long-term injuries in AKAP12-KO mice included the increase in ductular proliferation and liver cancer formation, respectively. These results indicate that AKAP12 may mediate intercellular communication between sinusoidal endothelial cells and cells with epithelial characteristics. Moreover, sinusoidal AKAP12 expression was reduced following liver injury, indicative of the existence of a mechanism that alters the expression level of AKAP12 in sinusoidal endothelium.

Previous studies have highlighted the downregulation of AKAP12 expression in human HCC, suggesting its possible role as a tumor suppressor in HCC (13,15,16). Several mechanisms have been proposed to be involved in the reduction of AKAP12 expression in HCC. Goepfert *et al* demonstrated that AKAP12 reduction in advanced cancer was associated with chromosomal loss and promoter hypermethylation and revealed MiR-183/186-dependent regulation of AKAP12 expression in cirrhosis and dysplastic nodules (15). Hayashi *et al* showed AKAP12 reduction in HCC by hypermethylation and without chromosomal deletion (16), whereas Xia *et al* proposed MiR-103-mediated regulation of AKAP12 expression as a mechanism of HCC development (13). Therefore, multiple mechanisms may be involved in the downregulation of AKAP12 expression in HCC. Considering our data demonstrating prominent AKAP12 expression in liver sinusoids, we suggest that some of the downregulation mechanisms may be responsible for the reduction in sinusoidal AKAP12 expression in injured livers. Future studies with isolated tumor cells and tumor-associated endothelial cells are necessary to address the cell-type specific mechanism underlying AKAP12 reduction in HCC.

Liver fibrosis and the development of HCC are linked pathogenesis as most HCCs develop under fibrotic background (3). Therefore, the effect of AKAP12 deficiency on HCC development could be caused by its earlier effects on liver fibrosis. When we addressed the effect of AKAP12 deficiency on liver fibrosis by short term TAA treatment, we did not observe significant changes in overall ECM accumulation as revealed by picro-sirius red staining (Fig. 3A). Interestingly, however, transcript levels of specific ECM genes were elevated in AKAP12 KO livers (Fig. 4A), suggesting that AKAP12-deficiency led to the alteration in some fibrosis-related gene expression by hepatic myofibroblasts. The discrepancy between transcript levels and histological ECM accumulation is probable owing to the complexity of ECM remodeling during fibrogenesis. Under fibrotic circumstances, liver cells produce not only ECM proteins but also matrix metalloproteases (MMPs) and tissue inhibitor of metalloproteases (TIMPs), and therefore the tissue level of ECM accumulation is determined by the balance between ECM proteins and ECM-modifying enzymes (31). Thus, it is possible that AKAP12-mediated alteration in

certain ECM gene expression is diluted by ECM modifying enzymes. Further studies including whole transcriptome analysis of AKAP12-deficient livers will be able to address detailed mechanisms of AKAP12-mediated ECM remodeling.

Despite minor effect of AKAP12-deficiency on liver fibrosis, AKAP12 KO mice aggravated tumorigenesis after long term TAA administration (Fig. 5A). These results suggest AKAP12's additional tumor suppressing roles in tumorigenesis under fibrotic background. Considering specific AKAP12 expression by hepatic non-parenchymal cells, it may play tumor suppressor functions via the regulation of hepatic tumor microenvironment.

Taken together, our study suggests a novel mechanism underlying AKAP12-dependent inhibition of liver injuries that involves AKAP12 functions in hepatic non-parenchymal cells.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Global Core Research Center (GCRC) Program (grant no. 2011-00,30001), Bio & Medical Technology Development Program (grant no. 2015M3A9E6028949), and NRF grant (grant no. 2015R1C1A2A01054446, received by H.S. Lee) through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Ministry of Science, ICT and Future Planning (MSIP).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HSL designed and performed experiments, and wrote the manuscript. JC designed and performed experiments. TS performed statistical analysis. EJJ and JGK designed the experiments. SHR, DL, MKJ, EY and YHC performed human specimen preparation and analysis. IHG contributed to manuscript drafting and animal experiment data analysis. KWK designed the experiments and wrote the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. The human study was approved by the Institutional Review Board of the Asan Medical Center, Seoul, Korea. All mouse experiments were reviewed and approved by the Committee for Care and Use of Laboratory Animals at Seoul National University and performed according to the Guide for Animal Experiments edited by the Korean Academy for Medical Sciences.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Pellicoro A, Ramachandran P, Iredale JP and Fallowfield JA: Liver fibrosis and repair: Immune regulation of wound healing in a solid organ. *Nat Rev Immunol* 14: 181-194, 2014.
- Friedman SL: Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134: 1655-1669, 2008.
- Sakurai T and Kudo M: Molecular link between liver fibrosis and hepatocellular carcinoma. *Liver Cancer* 2: 365-366, 2013.
- Fausther M, Pritchard MT, Popov YV and Bridle K: Contribution of liver nonparenchymal cells to hepatic fibrosis: Interactions with the local microenvironment. *Biomed Res Int* 2017: 6824762, 2017.
- Carnegie GK, Means CK and Scott JD: A-kinase anchoring proteins: From protein complexes to physiology and disease. *IUBMB Life* 61: 394-406, 2009.
- Poppinga WJ, Muñoz-Llancao P, Gonzalez-Billault C and Schmidt M: A-kinase anchoring proteins: cAMP compartmentalization in neurodegenerative and obstructive pulmonary diseases. *Br J Pharmacol* 171: 5603-5623, 2014.
- Gelman IH: Suppression of tumor and metastasis progression through the scaffolding functions of SSeCKS/Gravin/AKAP12. *Cancer Metastasis Rev* 31: 493-500, 2012.
- Lee SW, Kim WJ, Choi YK, Song HS, Son MJ, Gelman IH, Kim YJ and Kim KW: SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier. *Nat Med* 9: 900-906, 2003.
- Liu W, Gong J, Hu J, Hu T, Sun Y, Du J, Sun C, Guan M, Jiang H and Lu Y: Quantitative assessment of AKAP12 promoter methylation in human prostate cancer using methylation-sensitive high-resolution melting: Correlation with gleason score. *Urology* 77: 1006.e1-7, 2011.
- Akakura S, Huang C, Nelson PJ, Foster B and Gelman IH: Loss of the SSeCKS/Gravin/AKAP12 gene results in prostatic hyperplasia. *Cancer Res* 68: 5096-5103, 2008.
- Gelman IH: The role of SSeCKS/gravin/AKAP12 scaffolding proteins in the spatiotemporal control of signaling pathways in oncogenesis and development. *Front Biosci* 7: d1782-d1797, 2002.
- Choi MC, Jong HS, Kim TY, Song SH, Lee DS, Lee JW, Kim TY, Kim NK and Bang YJ: AKAP12/Gravin is inactivated by epigenetic mechanism in human gastric carcinoma and shows growth suppressor activity. *Oncogene* 23: 7095-7103, 2004.
- Xia W, Unger P, Miller L, Nelson J and Gelman IH: The Src-suppressed C kinase substrate, SSeCKS, is a potential metastasis inhibitor in prostate cancer. *Cancer Res* 61: 5644-5651, 2001.
- Lin X and Gelman IH: Reexpression of the major protein kinase C substrate, SSeCKS, suppresses v-src-induced morphological transformation and tumorigenesis. *Cancer Res* 57: 2304-2312, 1997.
- Goepfert B, Schmezer P, Dutruel C, Oakes C, Renner M, Breinig M, Warth A, Vogel MN, Mittelbronn M, Mehrabi A, *et al*: Down-regulation of tumor suppressor A kinase anchor protein 12 in human hepatocarcinogenesis by epigenetic mechanisms. *Hepatology* 52: 2023-2033, 2010.
- Hayashi M, Nomoto S, Kanda M, Okamura Y, Nishikawa Y, Yamada S, Fujii T, Sugimoto H, Takeda S and Kodera Y: Identification of the A kinase anchor protein 12 (AKAP12) gene as a candidate tumor suppressor of hepatocellular carcinoma. *J Surg Oncol* 105: 381-386, 2012.
- Xia W, Ni J, Zhuang J, Qian L, Wang P and Wang J: MiR-103 regulates hepatocellular carcinoma growth by targeting AKAP12. *Int J Biochem Cell Biol* 71: 1-11, 2016.
- Liu Y, Meyer C, Xu C, Weng H, Hellerbrand C, ten Dijke P and Dooley S: Animal models of chronic liver diseases. *Am J Physiol Gastrointest Liver Physiol* 304: G449-G468, 2013.
- Wallace MC, Hamesch K, Lunova M, Kim Y, Weiskirchen R, Strnad P and Friedman SL: Standard operating procedures in experimental liver research: Thioacetamide model in mice and rats. *Lab Anim* 49 (1 Suppl): S21-S29, 2015.
- Abe M, Yoshida T, Akiba J, Ikezono Y, Wada F, Masuda A, Sakaue T, Tanaka T, Iwamoto H, Nakamura T, *et al*: STAT3 deficiency prevents hepatocarcinogenesis and promotes biliary proliferation in thioacetamide-induced liver injury. *World J Gastroenterol* 23: 6833-6844, 2017.
- Hartig SM: Basic image analysis and manipulation in ImageJ. *Curr Protoc Mol Biol Chapter* 14: Unit14 15, 2013. doi: 10.1002/0471142727.mb1415s102.
- Shin MW, Bae SJ, Wee HJ, Lee HJ, Ahn BJ, Le H, Lee EJ, Kim RH, Lee HS, Seo JH, *et al*: Ninjurin1 regulates lipopolysaccharide-induced inflammation through direct binding. *Int J Oncol* 48: 821-828, 2016.
- Sakurai T, Yada N, Watanabe T, Arizumi T, Hagiwara S, Ueshima K, Nishida N, Fujita J and Kudo M: Cold-inducible RNA-binding protein promotes the development of liver cancer. *Cancer Sci* 106: 352-358, 2015.
- Helmy SA, El-Mesery M, El-Karef A, Eissa LA and El Gayar AM: Chloroquine upregulates TRAIL/TRAILR2 expression and potentiates doxorubicin anti-tumor activity in thioacetamide-induced hepatocellular carcinoma model. *Chem Biol Interact* 279: 84-94, 2018.
- Balkwill FR, Capasso M and Hagemann T: The tumor microenvironment at a glance. *J Cell Sci* 125: 5591-5596, 2012.
- Hanahan D and Coussens LM: Accessories to the crime: Functions of cells recruited to the tumor microenvironment. *Cancer Cell* 21: 309-322, 2012.
- Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C and Marini FC: Tumor-associated stromal cells as key contributors to the tumor microenvironment. *Breast Cancer Res* 18: 84, 2016.
- Campbell I, Qiu W and Haviv I: Genetic changes in tumour microenvironments. *J Pathol* 223: 450-458, 2011.
- Fukino K, Shen L, Patocs A, Mutter GL and Eng C: Genomic instability within tumor stroma and clinicopathological characteristics of sporadic primary invasive breast carcinoma. *JAMA* 297: 2103-2111, 2007.
- Wernert N, Löcherbach C, Wellmann A, Behrens P and Hügel A: Presence of genetic alterations in microdissected stroma of human colon and breast cancers. *Anticancer Res* 21: 2259-2264, 2001.
- Arriazu E, Ruiz de Galarreta M, Cubero FJ, Varela-Rey M, Pérez de Obanos MP, Leung TM, Lopategi A, Benedicto A, Abraham-Enachescu I and Nieto N: Extracellular matrix and liver disease. *Antioxid Redox Signal* 21: 1078-1097, 2014.



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