

ADAM17 promotes the metastasis of hepatocellular carcinoma via upregulation MMP21

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Abstract

Background

The upregulation of ADAM17 has been reported to be associated with invasion and metastasis in various tumors, however the molecular mechanism of ADAM17 in the progression of hepatocellular carcinoma (HCC) remain to be clarified. Human matrix metalloproteinase 21 (MMP21), the newest member of the MMP gene family, has been suggested to play an important role in embryogenesis and tumor progression. So far, nothing is known about the relationship between ADAM17 and MMP21.

Methods

In this study, the expression level of ADAM17 and MMP21 in HCC tissues was measured by immunohistochemistry. The Scratch wounding assay and Transwell were used to identify the invasion and metastasis ability. ELISA was used to evaluate the production of MMP21. Coimmunoprecipitation experiments demonstrated a direct association between ADAM17 and MMP21. HPLC was used to confirmed that ADAM17 participated in the maturation of MMP21.

Results

Our present data indicated that ADAM17 and MMP21 was significantly upregulated in human HCC tissues. Knockdown of ADAM17 in HCC inhibited cell invasion and metastasis. Moreover, ADAM17 regulates the secretion and expression of MMP21. Furthermore we discovered a direct association between ADAM17 and MMP21, and we also found MMP21 prodomain could be cleaved by ADAM17.

Conclusion

Our data suggest that ADAM17 plays an important role in the development of HCC invasion and metastasis and this function may be implement by MMP21.

Background

Hepatocellular carcinoma (HCC) is a highly aggressive and heterogeneous disease. The latest national cancer statistics released by the National Cancer Center showed that the morbidity of hepatocellular carcinoma is 36.5 per 10⁴ people and that it ranks fourth in malignant tumors [1,2]. Therefore, we explored the mechanisms underlying the carcinogenesis and progression of HCC to benefit therapy.

ADAM17 was initially identified as an important member of the ADAM family by Black RA's team in 1997. Because the enzyme is responsible for releasing soluble tumor necrosis factor-alpha (TNF α) from the plasmalemma, it is also known as TNF α converting enzyme (TACE/ADAM17)[3]. It has been reported that the dysregulation of ADAM17 contributes to the pathology of various cancers. For instance, ADAM17 protein was highly expressed in esophageal squamous cell carcinoma (ESCC) and promoted the development, invasion and metastasis of ESCC[4-7]. Similarly, ADAM17 silencing suppressed the

invasion and proliferation of MCF7 cells in vitro[8]. Furthermore, Wang H P's study suggested that Nox1 promoted colorectal cancer metastasis by stabilizing ADAM17[9]. Although several studies have shown that ADAM17 promotes the occurrence and development of hepatocellular carcinoma[10,11], the potential regulatory mechanism has not been fully elucidated.

MMP21 is the last uncharacterized MMP which is different from other MMPs and is rarely produced in normal tissues without inflammatory or oncogenic stimuli, which may implicate a role for MMP21 in normal tissue homeostasis [10,11]. The prodomain of MMP21 contains a peptide sequence similar to that of TNF α . It has also been reported that MMP21 is expressed in cancer cells located in the invasive front of tumors rather than dysplastic cells and enhances tumor metastasis in some solid tumors[12-15]. Positive correlations between MMP21 and tumor diameter, depth of invasion, vessel invasion, lymph node distant metastases, and tumor-node-metastasis stage were observed in gastric cancer. The overall survival rate was significantly lower in MMP21- and MMP28- positive patients[15]. However, the expression pattern of MMP21 in HCC and whether MMP21 could be activated by other proteinases remain unknown. Notably, in this study, we investigated whether ADAM17 may regulate the maturation of MMP21 and influence the progression of HCC.

Methods

Patients and specimens

All experimental procedures involving the use of human tissue included the relevant receipt of written informed consent and were approved by the institutional review board at The XiangYa Hospital of Centre South University. For formalin-fixed paraffin-embedded HCC samples, human specimens were collected from the XiangYa Hospital tissue biobank, and the protocol for staining was approved by the local ethics committee of The XiangYa Hospital.

Immunohistochemistry and evaluation of immunostaining intensity

The tumor tissue was subjected to immunohistochemistry and stained with primary antibodies against ADAM17 (Abcam, ab2051, 1:100) and MMP21 (Abcam, MAB2079Z, 1:200) followed by light microscopy examination. The immunostaining intensity for the two proteins was reviewed and independently scored by pathologists who were blinded to the clinical data and scored independently according to the staining intensity and the proportion of stained tumor cells. According to the staining intensity, samples were scored as follows: no staining = 0; light yellow (weak staining) = 1; yellow brown (moderate staining) = 2; and brown (strong staining) = 3. The scores were expressed in terms of the proportion of cell staining as follows: scores of 0, 1, 2, and 3 indicated 0, $\leq 30\%$, 30%-70% and $\geq 70\%$ positive cells, respectively. Thus, the two combined scores (from the two independent pathologists) were taken as the final score, where 0 indicated negative (-); 1-2, weak positive (+); 3-4, strong positive (++); and 5-6, very strong positive (+++). In the statistical analyses, (+ +) and (+ + +) were classified as the positive group, while (-) and (+) were classified as the negative group.

Cell culture

Human HCC MHCC97H and Huh7 cells were cultured in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. Human HCC SMMC7721 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Cell invasion assay

For the transwell invasion assay, 24-well transwell units with an 8-µm pore size polycarbonate filter (Millipore) were used according to the manufacturer's instructions. Briefly, filters were coated with Matrigel to form a continuous thin layer. Then, cells were seeded in DMEM in the upper chamber. The lower chamber was filled with DMEM with 10% FBS. Following 24 h of incubation at 37 °C, cells remaining in the upper compartment were removed using cotton swabs. The cells that invaded through the filter into the lower compartment were fixed with 4% paraformaldehyde and stained with crystal violet (0.5% in 20% methanol). To quantify invasive cells, three independent fields of invasive cells per well were photographed.

Scratch wounding assay

Transfected cells were plated in six-well plates and incubated at 37 °C until a confluent monolayer was formed (> 90%). With a 100-µl sterile pipette tip, a scratch was created. The cells were washed three times with PBS (pH 7.2) to remove cell fragments, and low serum DMEM was added. Micrographs were taken immediately after wounding and after 24 h, 48 h, 72 h, and 110 h. The healing rate was calculated using the following equation: healing rate = $[1 - (T_x/T_0)] \times 100\%$, where T₀ is the wounded area at 0 h and T_x is the wounded area after x h.

Western blot

Cells were then harvested in lysis buffer. A BCA protein assay kit was used to determine the concentration of protein. Samples were separated on a 10% SDS-PAGE gel, followed by transfer to polyvinylidene difluoride (PVDF) membranes in an electrophoretic manner. The primary antibodies were used at a 1:1000 dilution, the loading control anti-tubulin was used at a 1:5000 dilution, and the secondary antibody was used at a 1:1000 dilution. The targeted proteins in the membrane were detected with an electrochemiluminescence detection system followed by exposure to X-ray film.

ELISA

To evaluate the production of MMP21 substrates, the transfected cells were prepared. Relevant ELISA kits were used to analyze the collected medium specimens for the proteins of interest. Supernatants were collected in triplicate for each cell line.

Immunoprecipitation

Cells underwent cytolysis in 1 ml of RIPA buffer, followed by a 10-min incubation on ice. Total cell lysates were centrifuged for 10 min at 20,000xg at 4 °C. Ten microliters of primary antibody was used to incubate the supernatants for 60 min. Then, 20 µl of protein A/G PLUS-agarose was added to the lysate and incubated at 4 °C overnight. The beads were washed with ice-cold RIPA buffer four times. The samples were then suspended and denatured in SDS sample buffer (which contained 100 mM dithiothreitol, 10% glycerol, 50 mM Tris, pH 6.8, 2% SDS, and 0.01% bromophenol blue).

HPLC method

Recombinant human TACE/ADAM17 (rhTACE) (Catalog # 930-ADB) was purchased from R&D Systems. The amino acid polypeptide sequence (ALAQAVRRFQ) was ordered from Sangon Biotech. The assay buffer used in this study was 25 mM Tris, 2.5 µM ZnCl₂, 0.005% Brij-35 (w/v), pH 9.0. First, rhTACE was diluted to 0.2 ng/µl in assay buffer. Second, the substrate was diluted to 20 µM in assay buffer. Third, 50 µl of 0.2 ng/µl rhTACE was added, and the reaction was initiated by adding 50 µl of 20 µM substrate. A substrate blank was included that contained 50 µl of assay buffer and 50 µl of substrate. The HPLC reaction conditions in our study were as follows: column: 250*4.6 mm, Sinchrom ODS-BP-5; solvent A: 0.1% TFA in 100% water; solvent B: 0.1% TFA in 100% acetonitrile; flow rate: 1.0 ml/min; wavelength (nm): 220; and volume: 10 µl.

Results

ADAM17 and MMP21 are significantly upregulated in human HCC tissues, and there is a positive correlation between the two genes.

To examine the expression of ADAM17 and MMP21 in patients with HCC. First, we analyzed the UALCAN database and found that the expression level of ADAM17 in primary tumors was higher than that in normal tissues (Fig. 1A). Subsequently, we analyzed the expression of MMP21, and the result was consistent with ADAM17 (Fig. 1B). To further confirm this discovery, we performed immunohistochemistry to investigate the expression of ADAM17 and MMP21 in human HCC tissues and adjacent tissues (Fig. 1C). As shown in Fig. 1C and Fig. 1D, the expression levels of ADAM17 and MMP21 were both higher in HCC tissues than in adjacent liver tissues ($P < 0.001$) (Table 1). Our statistical analysis showed that there was a positive correlation between ADAM17 and MMP21 in HCC (Table 2). Furthermore, we evaluated the correlation of ADAM17 and MMP21 with the patients' clinicopathological parameters and found that the expression of MMP21 was related to the patient's microvascular invasion, and the results were statistically significant (Table 3). Although there was no significant correlation between ADAM17 and other clinicopathological parameters in HCC, the expression of ADAM17 may be correlated with microvascular invasion, as the P-value is 0.063. In conclusion, our results suggest that ADAM17 and MMP21 may play an important role in the development of HCC.

ADAM17 promotes the viability and migration of hepatocellular carcinoma.

To assess the role of ADAM17 in the invasion and migration of hepatocellular carcinoma. We silenced ADAM17 in HCC cells (MHCC97H, SMMC7721) using small interfering RNAs (siRNAs). We found that silencing ADAM17 obviously changed the cell morphology (Fig. 2A). To determine whether ADAM17 is associated with cell motility and metastasis, we performed invasion and wound healing assays. The number of migrated MHCC97H and SMMC7721 cells was significantly decreased following ADAM17 silencing ($P < 0.05$; Fig. 2B, 2C). Furthermore, in the wound healing assay, we confirmed that the ranges of healing rates were reduced by ADAM17 downregulation in MHCC97H and SMMC7721 cells. Our results confirm that ADAM17 plays a pivotal role in hepatocellular carcinoma motility and migration.

The expression of MMP21 in HCC cells is associated with ADAM17.

Studies have shown that MHCC97H has the highest metastatic potential compared to other hepatocellular carcinoma cells. We examined MMP21 expression in three kinds of hepatocellular carcinoma cells by western blot (Fig. 3A). Our data indicate that the expression of MMP21 is significantly higher in MHCC97H cells than in Huh7 and SMMC7721 cells, which have lower metastatic potential, suggesting that MMP21 may be associated with tumor cell metastasis. Immunohistochemistry analysis showed a positive correlation between ADAM17 and MMP21 in patients with liver cancer (Table 2). To determine whether ADAM17 has an effect on MMP21 expression and activity, we first used siRNA to knockdown ADAM17 in Huh7 and SMMC7721 cells. As shown in Fig. 3B and Fig. 3C, we observed that the expression of mMMP21 was significantly downregulated in the siRNA group (Huh7 and SMMC7721) in comparison with expression in the control. In addition, we also overexpressed ADAM17 in Huh7 and SMMC7721 cells, and the expression of mMMP21 was markedly increased in the ADAM17 overexpression group compared with that in the control (Fig. 3D, 3E). This demonstrated that ADAM17 can regulate the expression of MMP21.

ADAM17 expression influences mMMP21 secretion.

Next, we investigated MMP21 secretion by ELISA. In Huh7 cells, supernatant from ADAM17 overexpression (pcDNA3.1(+)-ADAM17) or control (pcDNA3.1(+)) conditions was harvested for measurements of MMP21 secretion over a period of 48 and 72 h after transfection without changing the media. We found that MMP21 secretion increased in pcDNA3.1(+)-ADAM17 transfectants at both time points (48 h and 72 h) (Fig. 4A), and the secretion ratio increased significantly compared with the control at 48 h, but there was no difference at 72 h. Furthermore, MMP21 secretion with siRNA-ADAM17 or control transfection in Huh7 cells was detected. The secretion difference between the two groups was remarkable at 48 h and 72 h (Fig. 4B). To further verify the results, we also tested MMP21 secretion levels in MHCC97H cells. The overexpression of ADAM17 enhanced the MMP21 secretion level at both 48 h and 72 h ($P < 0.05$, Fig. 4C). However, there was no significant difference in MMP21 secretion in MHCC97H cells when ADAM17 was knocked down (Fig. 4D). These data confirmed that ADAM17 influences mMMP21 secretion, but not just ADAM17 can influence the secretion of mMMP21.

ADAM17 and MMP21 interact with each other in Huh7 cells, and the prodomain of MMP21 may be cleaved by ADAM17.

The IP approach was applied to further prove the correlation between MMP21 and ADAM17. As shown in Fig. 5A, ADAM17 and MMP21 exhibited cross-linking in Huh7 cells. TNF α , a novel substrate for ADAM17 can be cleaved at a special site, Pro-Leu-Ala-Gln-Ala-|-Val-Arg-Ser-Ser-Se. Interestingly, the predomain of MMP21 contains a similar amino acid sequence, Leu-Ala-Gln-Ala-Val-Arg. Thus, we prepared a peptide (ALAEAVRRFQ) representing the sequence of pro-MMP21 and recombinant adam17 (rhTACE). The synthesized peptide was incubated with purified protease rhTACE, and then products were analyzed by reverse phase HPLC. As the data show, two main peaks were observed in the experimental group, while there was only one main peak in the control group (Fig. 5B, 5C), and the enzyme cleaved the peptide. Taken together, the results confirmed that ADAM17 participated in the maturation of MMP21.

Discussion

HCC is one of the most prevalent human cancers. Tumor invasion is the main cause of mortality in patients with HCC[16–19]. Recent studies have determined that ADAM17 dysfunction may play an important role in tumor invasion[20]. In 2016, CD133-expressing CSCs were shown to be responsible for cell invasion and migration after radiation, and their radiation-induced metastatic potential could be prevented by suppression of ADAM17[21]. A recent study proved that ADAM17 promotes cell invasion and migration through the integrin β 1 pathway in HCC[22]. In our study, we first used a database to confirm that ADAM17 was correlated with the OS and PFS of patients with HCC. Later, we further identified that ADAM17 was markedly increased in HCC tissue samples. Moreover, we found that downregulation of ADAM17 could significantly suppress the invasion of MHCC97H and SMCC7721 cells. Therefore, ADAM17 may represent a novel target in the progression of HCC.

ADAM17 was originally identified as an enzyme responsible for processing TNF α from a precursor to a soluble circulating form[3, 23]. As a protein cleaved by ADAM17, TNF α seems to lack a definable consensus cleavage motif[19]. However, statistics in the MEROPS database showed that there is orderliness. It is more selective for alanine at the P1 position and revealed a preference for valine at the P1' position among 60 cleavage sites registered in this database. Recently, some studies demonstrated that cleavage site specificities were in excellent agreement with the information derived from the MEROPS database[24,25]. Interestingly, compared with the cleavage peptide (LAQAVRSS), we found that the prodomain of MMP21 has a similar peptide sequence (LAQAVR) through the Swiss Prot database. Furthermore, MMP21 was recently shown to play an important role in tumor processing[14–16, 26]. In our study, MMP21 was found to be upregulated in hepatocellular carcinoma and associated with microvascular invasion. In addition, the high expression of MMP21 in MHCC97H cells is often accompanied by high metastatic potential. Importantly, downregulation of ADAM17 could decrease the expression of MMP21, while overexpression of ADAM17 can markedly increase the expression of MMP21. Furthermore, ADAM17 can positively regulate the secretion of MMP21. Next, we confirmed that ADAM17 coimmunoprecipitated with MMP21 in the Huh7 cell line. On the other hand, it was further indicated by high-performance liquid chromatography that the precursor of MMP21 may be cleaved by ADAM17 protease.

Conclusion

Our study demonstrated that increased ADAM17 expression may have contributed to HCC metastasis and progression. We found that ADAM17 and MMP21 expression in HCC was significantly higher than that in normal liver tissues and was associated with microvascular invasion both in vivo and in vitro. We first showed that ADAM17 was positively correlated with MMP21 in HCC. Overexpression of ADAM17 may improve tumor processing by cleaving the prodomain of MMP21 and activating it. In our study, we further expose the new mechanism of ADAM17 in HCC metastasis. Thus, ADAM17 is likely to be an attractive target for inhibiting HCC metastasis.

Declarations

Availability of data and materials

The data and materials used to support the findings of this study are available from the corresponding author upon request.

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Contributions

Xiang Yuqi: Cell culture, Cell invasion assay, Scratch wounding assay, Western blot, ELISA, Immunoprecipitation, HPLC, IHC, Write article. Liu Liyu: Cell culture, Western blot, Statistic analysis, Database analysis, Data arrangement, Write article. Wang Ying: IHC data analysis, Li Bo: IHC data

analysis. Peng Jinwu: IHC data analysis, Subject design and guidance. Feng Deyun: Subject design and guidance, Funds support.

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Ethics declarations

All experimental procedures involving the use of human tissue included the relevant receipt of written informed consent and were approved by the institutional review board at The XiangYa Hospital of Centre South University. For formalin-fixed paraffin-embedded HCC samples, human specimens were collected from the XiangYa Hospital tissue biobank, and the protocol for staining was approved by the local ethics committee of The XiangYa Hospital.

Ethics approval and consent to participate Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Additional information

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Tables

Due to technical limitations, Tables 1-3 are provided in the Supplementary Files section.

Figures

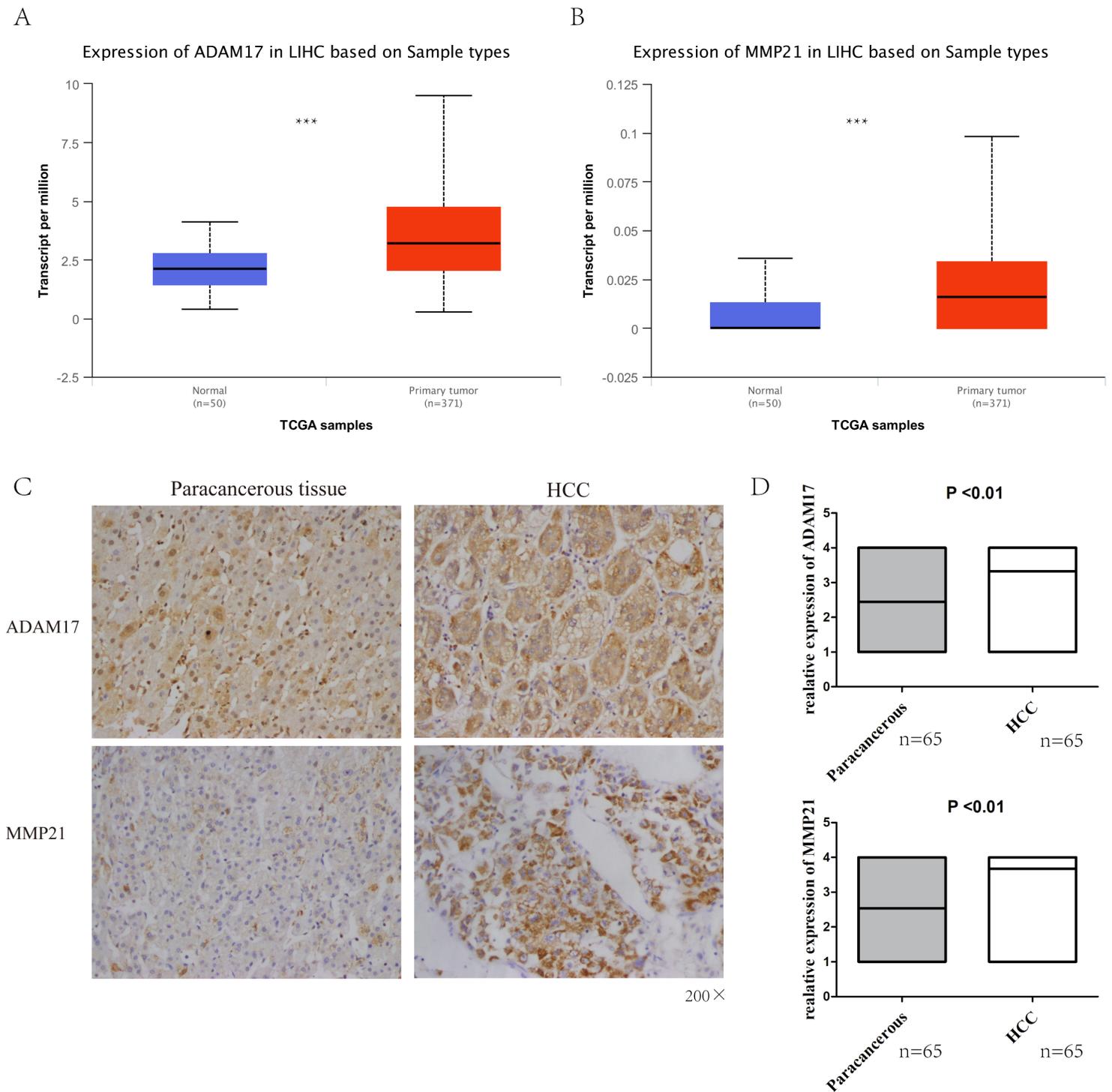


Figure 1

Levels of ADAM17 and MMP21 in patients with HCC. (A) High expression of ADAM17 in tumor tissue of patients with HCC compared with normal tissue in TCGA samples. (B) The high expression of MMP21 in

tumor tissue of patients with HCC compared with normal tissue in TCGA samples. (C) Representative micrographs of immunohistochemical staining of ADAM17 and MMP21 in HCC tissues and adjacent liver tissue are shown. (D) Expression levels of ADAM17 and MMP21 in HCC tissues (n=65) and adjacent liver tissue (n = 65) (t-test).

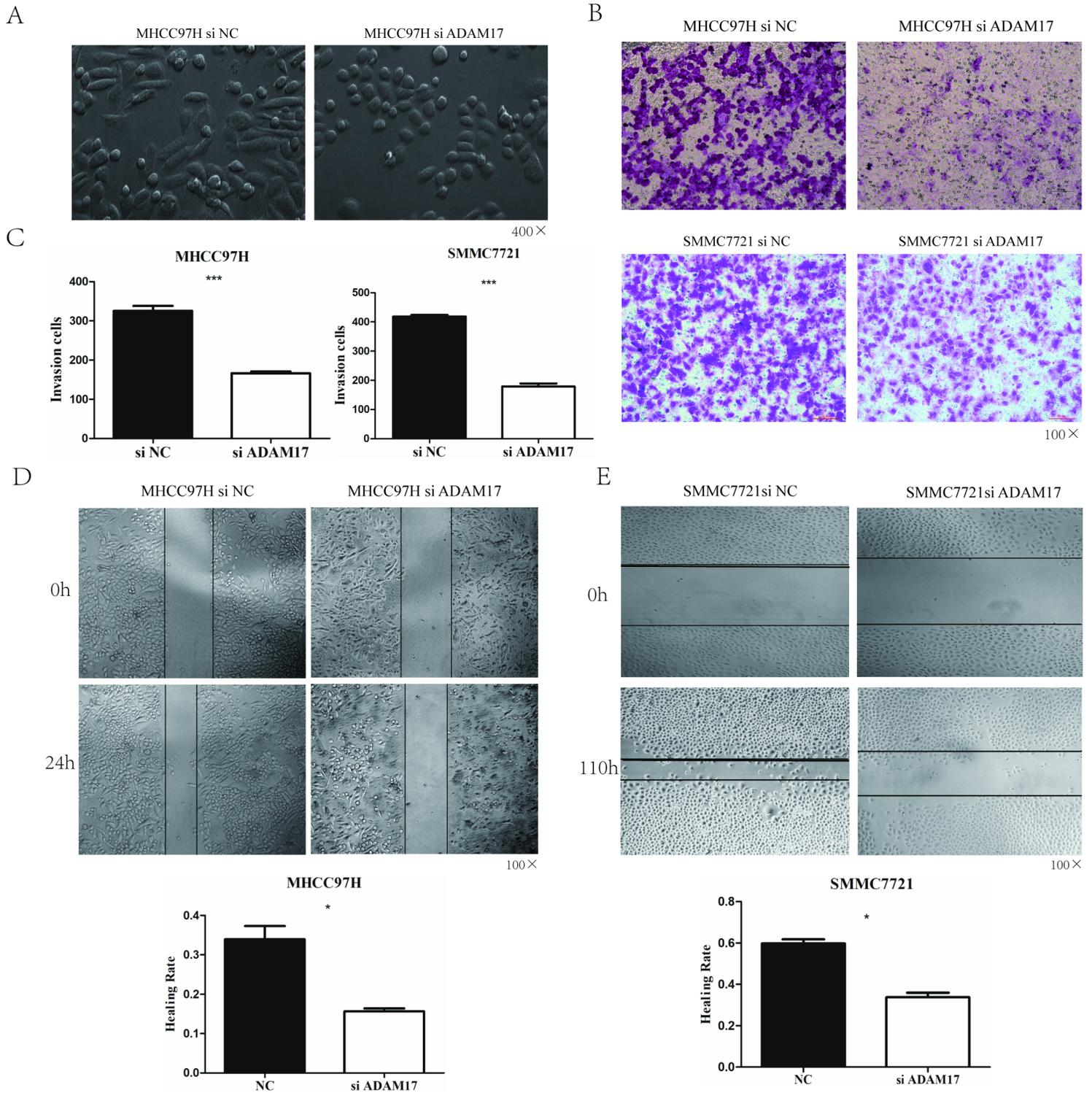
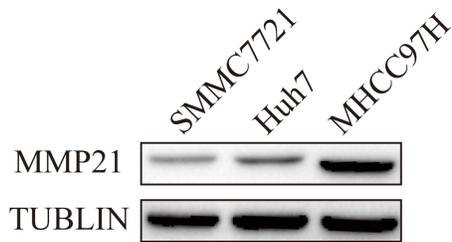


Figure 2

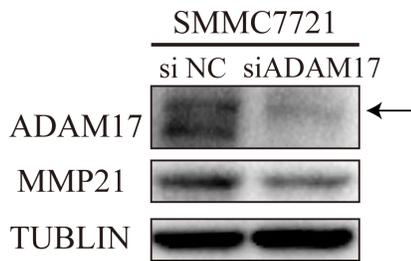
ADAM17 promotes the viability and migration of hepatocellular carcinoma cells. (A) Morphological changes in MHCC97H cells after knockdown of ADAM17. (B) Knockdown of ADAM17 impairs the

migratory and invasive capacities of HCC cells. MHCC97H and SMMC7721 migratory cells are shown. (C) Quantification of cell invasion through Matrigel with each cell treatment (***P*<0.001 based on the Mann-Whitney U test). (D, E) Effects of ADAM17 suppression on cell viability and migration measured by the wound healing assay in MHCC97H and SMMC7721 cells (magnification, ×100).

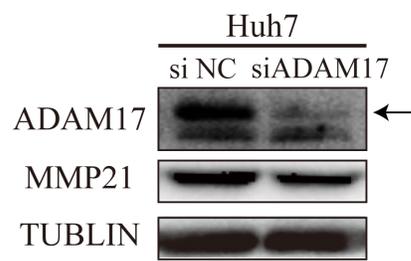
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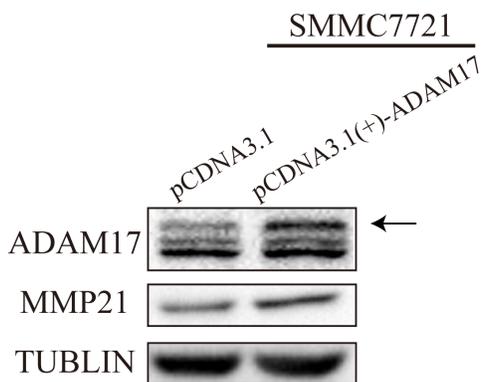
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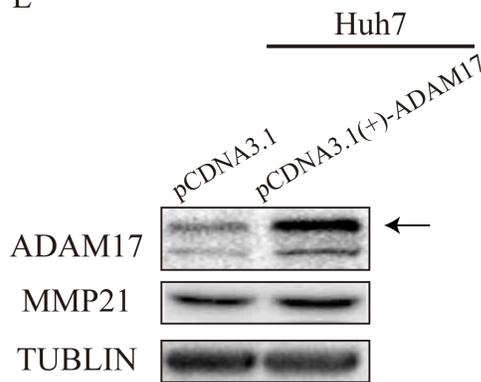


Figure 3

Knockdown and overexpression of ADAM17 in HCC cells affect the level of mature MMP21. (A) Expression of mMMP21 in SMMC7721, Huh7 and MHCC97H cells. (B, C) The expression of mature MMP21 in SMMC7721 and Huh7 cells treated with ADAM17 siRNA. (D, E) The expression of mature MMP21 in SMMC7721 and Huh7 cells treated with pCDNA3.1(+)-ADAM17.

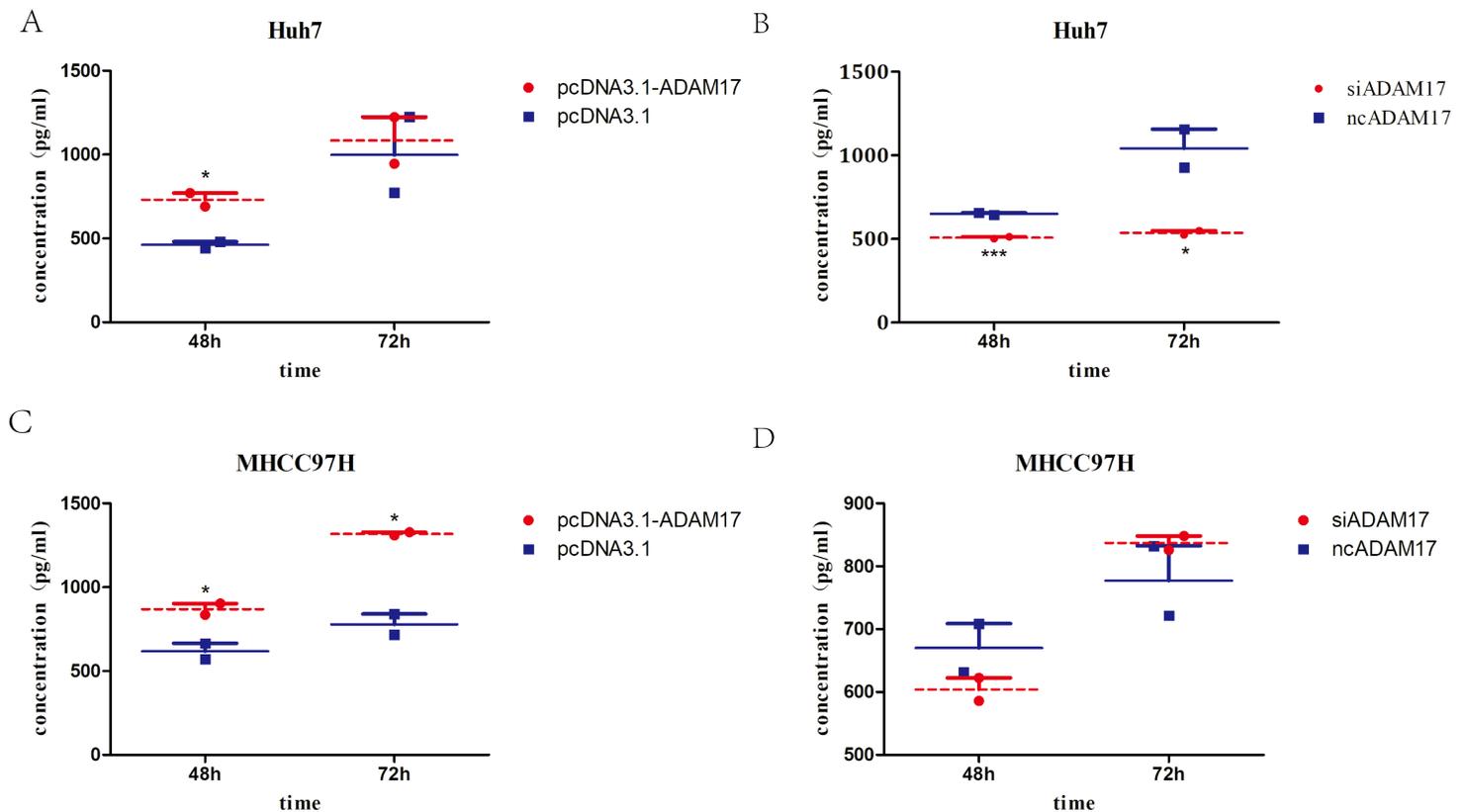
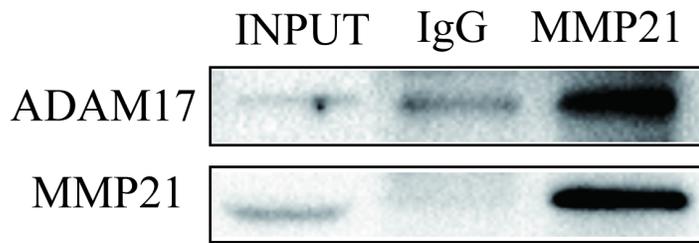


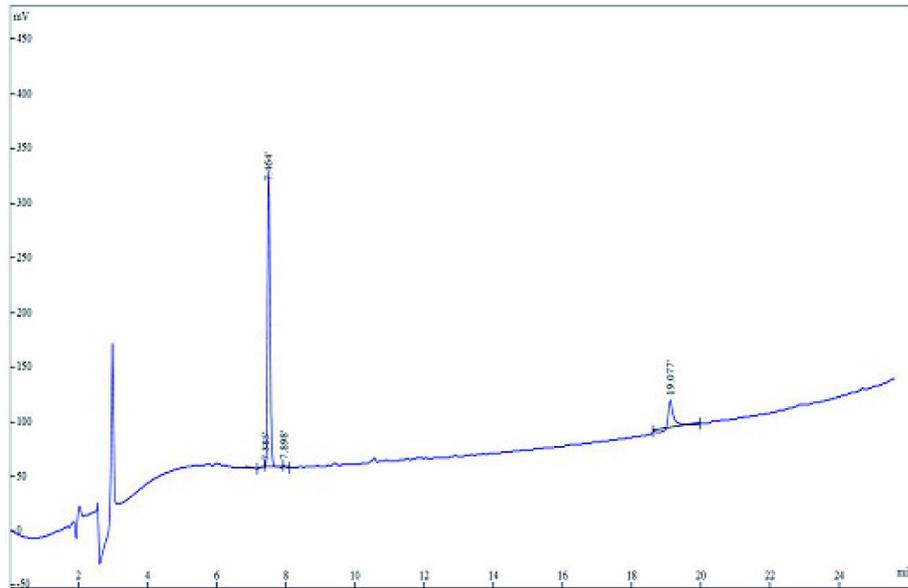
Figure 4

The secretion level of MMP21 in HCC cells. (A) Overexpression of ADAM17 enhanced the MMP21 secretion level in MHCC97H cells. (B) Knockdown of ADAM17 reduced MMP21 secretion in MHCC97H cells. (C) Overexpression of ADAM17 enhanced the MMP21 secretion level in Huh7 cells. (D) Knockdown of ADAM17 reduced MMP21 secretion in Huh7 cells (** $P < 0.01$, * $P < 0.05$).

A



B



C

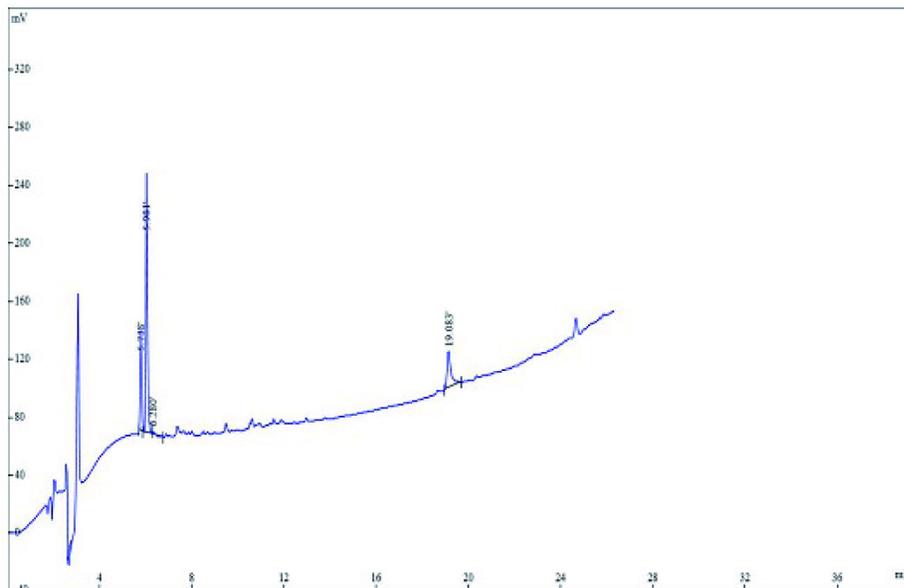


Figure 5

ADAM17 and MMP21 interact with each other in Huh7 cells, and the prodomain of MMP21 may be cleaved by ADAM17. (A) Immunoprecipitation analysis of the association between ADAM17 and MMP21 in Huh7 cells. (B) Analysis of a mixture of pro-MMP21 peptide with reaction buffer by UPLC-MS. (C) Analysis of a mixture of pro-MMP21 peptide with recombinant adam17 by UPLC-MS.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tables.pdf](#)