

ORIGINAL ARTICLE

Anti-CD47 antibody suppresses tumour growth and augments the effect of chemotherapy treatment in hepatocellular carcinoma

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Keywords

CD47 – chemoresistance – HCC

Abbreviations

CFSE, carboxyfluorescein succinimidyl ester; HCC, hepatocellular carcinoma; PDTX, patient-derived xenograft; SIRP- α , signal regulatory protein-alpha; T-IC, tumour-initiating cell; TSP-1, thrombospondin-1.

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide (1) and is often associated with metastasis and recurrence, even after surgical resection, leading to a poor prognosis. Therefore, the development of novel treatment regimens is urgently needed to improve the survival of these patients.

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Abstract

Background & Aims: Hepatocellular carcinoma (HCC) is often associated with metastasis and recurrence leading to a poor prognosis. Therefore, development of novel treatment regimens is urgently needed to improve the survival of HCC patients. In this study, we aimed to investigate the *in vitro* and *in vivo* effects of anti-CD47 antibody alone and in combination with chemotherapy in HCC. **Methods:** In this study, we examined the functional effects of anti-CD47 antibody (B6H12) on cell proliferation, sphere formation, migration and invasion, chemosensitivity, macrophage-mediated phagocytosis and tumourigenicity both *in vitro* and *in vivo*. The therapeutic efficacy of anti-CD47 antibody alone or in combination with doxorubicin was examined in patient-derived HCC xenograft. **Results:** Blocking CD47 with anti-CD47 monoclonal antibody (B6H12) at 10 $\mu\text{g/ml}$ could suppress self-renewal, tumourigenicity and migration and invasion abilities of MHCC-97L and Huh-7 cells. Interestingly, anti-CD47 antibody synergized the effect of HCC cells to chemotherapeutic drugs including doxorubicin and cisplatin. Blockade of CD47 by anti-CD47 antibody induced macrophage-mediated phagocytosis. Using a patient-derived HCC xenograft mouse model, we found that anti-CD47 antibody (400 $\mu\text{g/mouse}$) in combination with doxorubicin (2 mg/kg) exerted maximal effects on tumour suppression, as compared with doxorubicin and anti-CD47 antibody alone. **Conclusions:** Anti-CD47 antibody treatment could complement chemotherapy which may be a promising therapeutic strategy for the treatment of HCC patients.

Accumulating evidence suggests the existence of tumour-initiating cells (T-ICs) in the perpetuation and treatment resistance of various cancers, including acute myeloid leukaemia (2), brain (3) and colon cancers (4). We have reported that in HCC, CD47 is preferentially expressed in liver T-IC and that its overexpression has clinical significance (5). Recently, anti-CD47 antibodies were found to induce macrophage-mediated phagocytosis of leukaemia, lymphoma and bladder cancer cells (6–8). In addition, these antibodies were also shown to significantly suppress tumour growth in xenograft

Key points box

- Anti-CD47 antibody suppresses the tumour-initiating properties of HCC cells.
- Anti-CD47 antibody suppresses the migration and invasion abilities of HCC cells.
- Anti-CD47 antibody induces macrophage-induced phagocytosis.
- Combination of anti-CD47 antibody and doxorubicin results in synergistic tumour suppression in a PDTX model.

mouse model studies of a wide range of cancers, including leiomyosarcoma, multiple myeloma, acute lymphoblastic leukaemia and various solid tumours (9–12). These studies emphasize the therapeutic role of anti-CD47 antibodies alone in the suppression of tumour growth through the induction of macrophage-mediated phagocytosis of various types of cancer. In addition, antagonizing CD47 rendered tumour cells susceptible to additional therapies and attack by the T-cell immune response (13). Thus far, there is only one report showing the therapeutic efficacy of anti-CD47 antibodies in HCC (14). However, it remains unknown whether CD47 antibodies would complement existing treatments, such as chemotherapy.

To examine the therapeutic role of anti-CD47 antibodies in HCC, we applied an anti-CD47 monoclonal antibody (B6H12) to high CD47-expressing HCC cells (MHCC-97L and Huh-7 cells). The mouse anti-CD47 antibody B6H12 suppressed the liver T-IC phenotype, including self-renewal, chemoresistance, invasiveness and tumorigenicity, in Huh-7 and MHCC-97L cells. In addition, this monoclonal antibody induced phagocytosis of MHCC-97L cells by macrophages *in vitro*. Using a patient-derived tumour xenograft (PDTX) model, the anti-CD47 antibody (B6H12) inhibited the growth of HCC cells and exerted a synergistic effect when combined with doxorubicin treatment. Our findings suggest that this anti-CD47 antibody in combination with doxorubicin may be a promising therapeutic strategy for the treatment of HCC.

Materials and methods**Cell lines**

Human HCC cell lines MHCC-97L (from Liver Cancer Institute, Fudan University, China) and Huh-7 (Japanese Cancer Research Bank, Tokyo, Japan) were maintained in DMEM with high glucose (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated foetal bovine serum (Gibco BRL), 100 mg/ml penicillin G, and 50 µg/ml streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO₂.

Proliferation assay

Cells at a density of 5×10^3 were seeded in 96-well cell culture plates and allowed to adhere overnight. At the indicated time points, cell proliferation was measured using the BrdU proliferation assay kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. The experiment was carried out three times independently.

Sphere formation assay

A total of 200 single HCC cells were plated in 24-well polyHEMA (Sigma)-coated plates. The cells were grown in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 0.25% methyl cellulose, supplemented with 4 µg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), B27 (Invitrogen), 20 ng/ml EGF (Sigma-Aldrich), and 20 ng/ml basic FGF (Invitrogen). In addition to these supplements, cells were either treated with 10 µg/ml anti-CD47 antibody or its control antibody. Cells were replenished with 50 µl of supplemented medium every second day. To propagate spheres *in vitro*, spheres were collected by gentle centrifugation and dissociated to single cells using TrypLE Express (Invitrogen). Following dissociation, trypsin inhibitor (Invitrogen) was used to neutralize the reaction, and the cells were cultured to generate the second generation of spheres. After 12–14 days, the developed spheroids with size over 100 µm were counted and their images were captured under light microscope.

Migration and invasion assays

A migration assay was performed as described (5). A cell invasion assay was performed with self-coated Matrigel (BD Biosciences, San Jose, CA, USA) on the upper surface of a transwell chamber. Cells that had invaded through the extracellular matrix layer to the lower surface of the membrane were fixed with 2% PFA in PBS and stained with crystal violet. Photographs of four randomly selected fields of the fixed cells were captured, and the cells were counted. The experiments were repeated independently three times.

Annexin V staining

Cells in binding buffer were stained with propidium iodide (PI) and FITC-conjugated Annexin V, as provided by the Annexin-V-FLUO+S Staining Kit (Roche Diagnostics) according to the manufacturer's instructions. The results were analysed using a Canto II flow cytometer and FACSDiva software (BD Biosciences).

Phagocytosis assay

Macrophages (5×10^4) were plated per well in a 24-well tissue-culture plate and incubated in serum-free

medium for 2 h. Tumour cells were labelled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's protocol (Invitrogen), and 5×10^4 CFSE-labelled live tumour cells were added to the macrophages. The indicated antibodies (10 $\mu\text{g}/\text{ml}$) were added and incubated for 2 h at 37°C. The macrophages were repeatedly washed and subsequently imaged with an inverted microscope (Leica DMI6000B). The phagocytic index was calculated as the number of phagocytosed CFSE+ cells per 100 macrophages. Phagocytosis was also determined using flow cytometric detection of CFSE +CD68+ macrophages.

Tumorigenicity assay

An *in vivo* evaluation of tumorigenicity was performed with NOD-SCID mice through tumour xenograft induction. Cells were suspended in 1:1 culture medium and BD Matrigel Matrix (BD Biosciences) and subcutaneously injected into the flanks of NOD-SCID mice, which were kept under observation. The tumours were harvested at the end of experiment for documentation.

Suppression of CD47 in HCC cells

For suppression of CD47 in HCC cells, lentiviral particles (DFCI-Broad RNAi Consortium, Boston) expressing shRNAs against human CD47 were used to downregulate CD47 mRNA (5). Transduced cells were selected with 2 $\mu\text{g}/\text{ml}$ puromycin.

In vivo therapeutic targeting and its combined effect with chemotherapy

Xenografts were established in 4- to 6-week-old NOD/SCID mice using PDTX#1. Treatment was started once the size of the xenograft reached approximately 4 \times 4 mm (length \times width). To evaluate the effect of the anti-CD47 antibody alone, two groups received the following daily administrations via intraperitoneal injection: (i) 400 $\mu\text{g}/\text{mouse}$ control IgG (MOPC-21) or (ii) 400 $\mu\text{g}/\text{mouse}$ anti-CD47 antibody (B6H12, BioXCell). For evaluation of the combined effect of CD47 suppression with conventional chemotherapy, an additional two groups were included, consisting of i.p. injections of either (iii) 2 mg/kg doxorubicin (EBEWE Phama, Unterach) plus control IgG (MOPC-21) or (iv) 2 mg/kg doxorubicin (EBEWE Phama, Unterach) plus the anti-CD47 antibody (B6H12, BioXCell). After 30 days of various treatments, the effects on tumour growth were measured and recorded.

Statistical analysis

The statistical significance of the results obtained for sphere formation and the invasion and migration assays was determined by Student's *t*-test using Microsoft Office Excel software (Microsoft Corporation,

Redmond, WA, USA). The results are shown as the means and standard deviations, and *P*-values less than 0.05 were considered statistically significant (**P* < 0.05, ***P* < 0.01).

Results

Anti-CD47 antibody suppresses of HCC cell self-renewal and tumourigenicity

A previous study demonstrated that the reduction in CD47 by a lentiviral-based knockdown approach suppressed the self-renewal and tumourigenicity of high CD47-expressing MHCC-97L and Huh-7 cells (5). To examine whether a similar effect is observed in HCC cells upon treatment with an anti-CD47 antibody (B6H12), we first examined the effect on HCC cell proliferation. Using a BrdU assay, we found no significant decrease in proliferation in MHCC-97L and Huh-7 cells upon treatment with the anti-CD47 antibody for 48 h at doses ranging from 1 $\mu\text{g}/\text{ml}$ to 10 $\mu\text{g}/\text{ml}$ (Fig. 1A). Next, we examined the effect of the anti-CD47 antibody on the initiation of tumour formation, which is the distinct property liver T-IC (14). Therefore, we first examined the effect of the anti-CD47 antibody on the tumour-initiation abilities of MHCC-97L and Huh-7 cells after pretreatment with 10 $\mu\text{g}/\text{ml}$ anti-CD47 antibody for 48 h using IgG as a control (Fig. 1B). The incidence of tumour formation was evaluated at 40 and 90 days after tumour cell inoculation of MHCC-97L and Huh-7 cells at a density of 500, 1000 and 5000. Compared with the control IgG, the HCC cells pretreated with the anti-CD47 antibody demonstrated decreased tumour-forming ability (Table S1). Some groups have previously demonstrated that one of the properties of T-ICs is their ability to survive under anchorage-independent conditions (15, 16). Therefore, we determined the effect of the anti-CD47 antibody at 10 $\mu\text{g}/\text{ml}$ on liver T-IC hepatosphere formation by MHCC-97L and Huh-7 cells, a dose that had no effect on cell proliferation (as shown in Fig. 1A). Compared to the control IgG (MOPC-21), we found that the anti-CD47 antibody at 10 $\mu\text{g}/\text{ml}$ significantly inhibited both primary and secondary hepatosphere formation of MHCC-97L- and Huh-7-derived cells (Fig. 1C).

Anti-CD47 antibody suppresses migration and invasion abilities of HCC cells

A previous study demonstrated that CD47⁺ HCC cells were more metastatic when compared with CD47⁻ cells and that the suppression of CD47 by shRNA approach inhibited the invasion and migration abilities of HCC cells (5). Thus, we next assessed whether the anti-CD47 antibody could suppress the invasion and migration abilities of HCC cells. For this purpose, we incubated MHCC-97L and Huh-7 cells with 2, 5 and 10 $\mu\text{g}/\text{ml}$ anti-CD47 antibody and subjected them to invasion and

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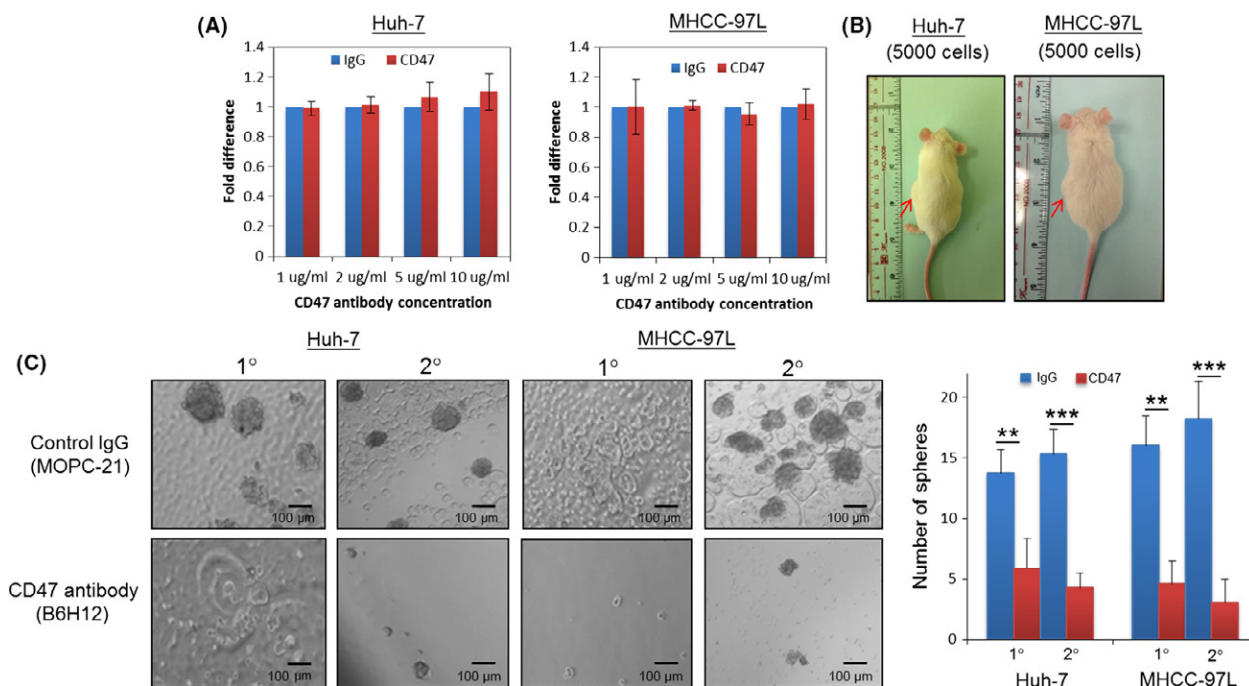


Fig. 1. Anti-CD47 antibody suppressed tumourigenicity and self-renewal in HCC cells. (A) Using a BrdU assay, the anti-CD47 antibody (B6H12) at the dose of 10 µg/ml had no significant effect on the proliferation of MHCC-97L and Huh-7 cells after incubation for 48 h. (B) Upon pretreatment of the anti-CD47 antibody for 48 h, the tumour-forming ability of Huh-7 and MHCC-97L cells was decreased (red arrows indicate tumour formation). The IgG-treated cells were injected into the left flank, while the anti-CD47 antibody-treated cells were injected into the right flank. (C) The anti-CD47 antibody at the dose of 10 µg/ml suppressed the size and number of hepatospheres derived from Huh-7 and MHCC-97L cells at their primary and secondary passages (** $P < 0.010$, *** $P < 0.001$, *t*-test). Error bars represent the standard deviation (SD) from at least three independent experiments.

migration assays. Compared with the control IgG (MOPC-21), we found that the anti-CD47 antibody suppressed the invasion and migration abilities of both MHCC-97L and Huh-7 cells, and the effect was found to be dose-dependent (Fig. 2).

Anti-CD47 antibody sensitizes HCC cells to chemotherapeutic drugs

The therapeutic role of anti-CD47 antibodies alone in suppressing tumour growth through the induction of macrophage-mediated phagocytosis has been demonstrated in various types of cancer. However, it remains unknown whether CD47 antibodies can complement the effect of chemotherapeutic drugs. Therefore, we examined whether B6H12 could sensitize HCC cells to the effect of chemotherapeutic drugs. For this purpose, we treated HCC cells with doxorubicin or cisplatin in combination with the anti-CD47 antibody to evaluate the effect on cell apoptosis by Annexin V staining. When compared with the control IgG, we found that the anti-CD47 antibody at 10 µg/ml significantly sensitized MHCC-97L cells to the effects of both cisplatin and doxorubicin (Fig. 3A). Similar effect was observed in Huh-7 cells (Fig. 3B), suggesting that the chemosensitization effect of the anti-CD47

antibody is not cell-type specific and macrophage-dependent.

Anti-CD47 antibody induces macrophage-mediated phagocytosis

Recently, the CD47-SIRP- α interaction was found to be a novel therapeutic target for human solid tumours (12). Thus, we examined the ability of clone B6H12 to induce the phagocytosis of MHCC-97L cells. In contrast to treatment with the isotype-matched mouse IgG (31.5%), MHCC-97L cells treated with the blocking antihuman CD47 antibody were efficiently phagocytosed by human macrophages (48.9%) (Fig. 4A). These results suggest that CD47 is an attractive therapeutic target and that a blocking anti-CD47 antibody may be an effective therapeutic agent to eliminate HCC cells. To further confirm whether CD47 indeed plays a crucial role in macrophage-mediated phagocytosis, we investigated the effect of CD47 knockdown on phagocytosis by comparing the degree of phagocytosis between shCD47 cells and control cells in the presence of human macrophages. Upon CD47 knockdown in Huh-7 cells, we found a significant increase in the number of phagocytosed cells when compared to the control cells (Fig. 4B).

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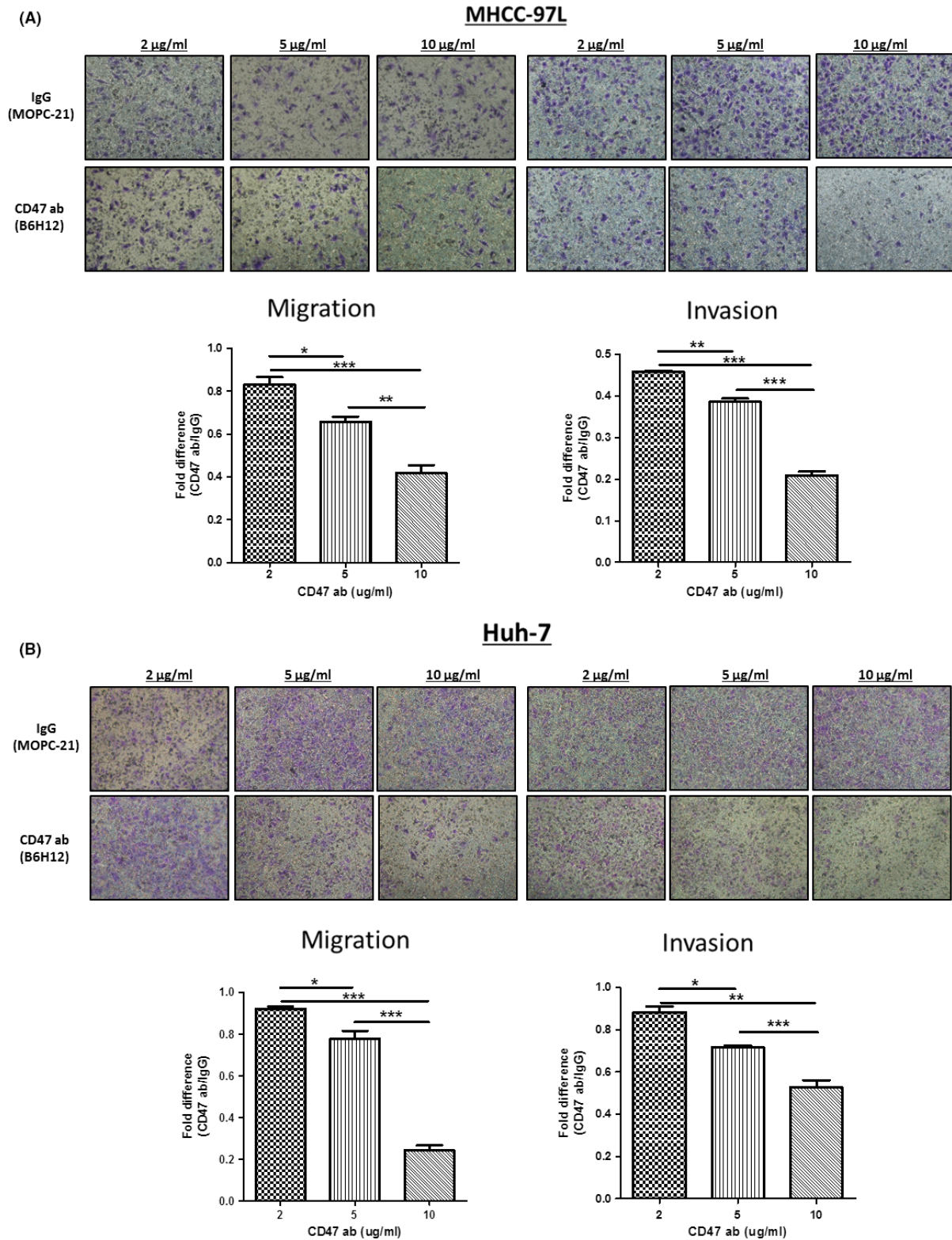


Fig. 2. Anti-CD47 antibody suppressed Huh-7 and MHCC-97L cell invasion and migration. The anti-CD47 antibody (B6H12) at the dose of 2, 5 and 10 µg/ml significantly reduced the invasive and migratory ability of (A) MHCC-97L (* $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$, t test) and (B) Huh-7 cells (* $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$, t test). Error bars represent the standard deviation (SD) from at least three independent experiments.

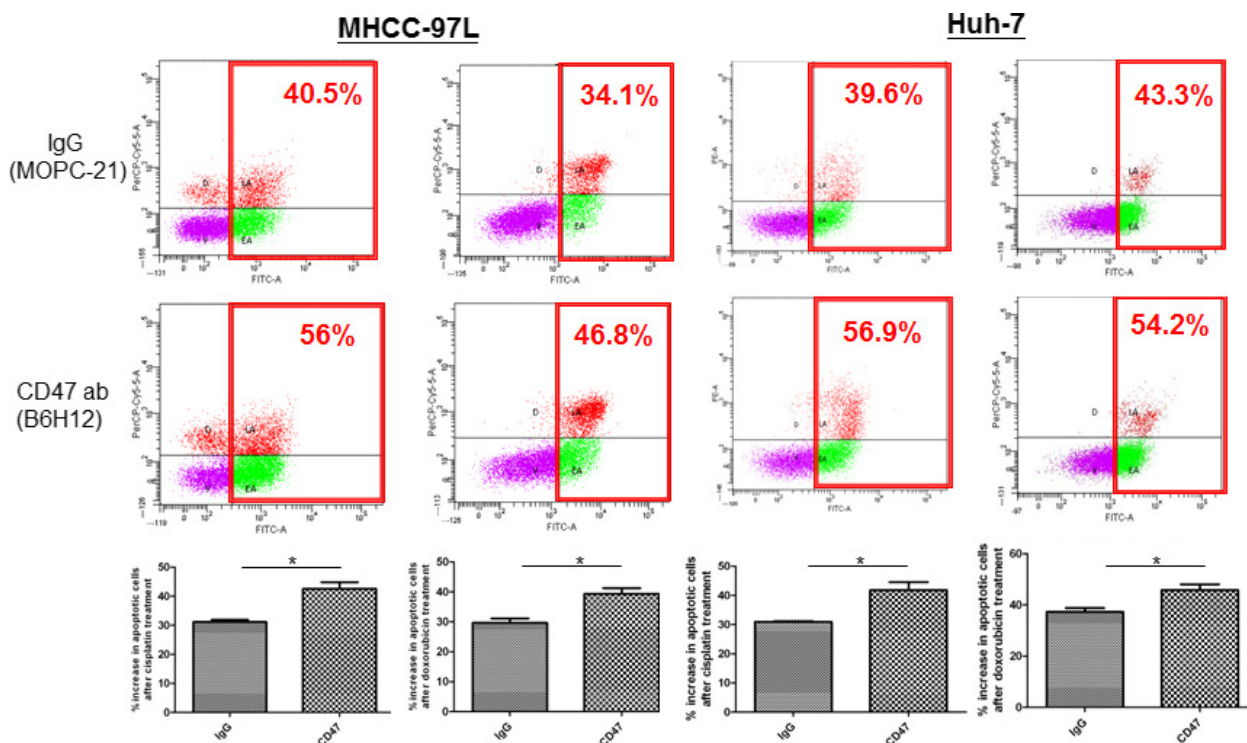


Fig. 3. Anti-CD47 antibody enhanced the chemosensitivity of Huh-7 and MHCC-97L cells. The anti-CD47 antibody (B6H12) at the dose of 10 $\mu\text{g/ml}$ significantly enhanced the effect of cisplatin and doxorubicin treatment on MHCC-97L cells ($P = 0.012$, $P = 0.0156$, t test); the anti-CD47 antibody (B6H12) at the dose of 10 $\mu\text{g/ml}$ also significantly enhanced the effect of cisplatin and doxorubicin treatment on Huh-7 cells ($P = 0.0163$, $P = 0.0175$, t test). Error bars represent the standard deviation (SD) from at least three independent experiments.

Combination of anti-CD47 antibody and doxorubicin results in synergistic tumour suppression in a PDTX model

We examined the therapeutic role of targeting CD47 alone and its combined effect with doxorubicin *in vivo* using PDTX#1 and the anti-CD47 monoclonal antibody. The following therapeutic regimens were used: (i) 400 $\mu\text{g/mouse}$ control IgG (MOPC-21), (ii) 400 $\mu\text{g/mouse}$ anti-CD47 antibody (B6H12, BioX-Cell), (iii) 2 mg/kg doxorubicin plus control IgG (MOPC-21) and (iv) 2 mg/kg doxorubicin plus the anti-CD47 antibody. Treatment was begun once the size of the xenografts had reached approximately 4 \times 4 mm. During the experiment, no signs of toxicity (infection, diarrhoea or loss of body weight) were observed in the animals undergoing anti-CD47 antibody treatment. The tumours and their corresponding volumes are shown in Fig. 5A,B. The anti-CD47 antibody suppressed tumour volumes in a manner similar to the effect of doxorubicin. In addition, the combination of anti-CD47 antibody and doxorubicin exerted a synergistic effect, resulting in maximal suppression of the tumours when compared with the control group.

Discussion

CD47 was reported to be a marker of T-ICs in leukaemia, HCC and bladder cancer (5, 7, 8). An anti-CD47 antibody (B6H12.2) preferentially enables the *in vitro* phagocytosis of human T-ICs derived from AML by macrophages, whereas a non-blocking anti-CD47 antibody failed to induce macrophage-mediated phagocytosis (7). A similar observation was found in colon $\text{ESA}^+\text{CD44}^+$ T-ICs (12). However, there is no report, thus far, showing the effect of an anti-CD47 antibody on the T-IC properties of cancer cells. In this study, we found that an anti-CD47 antibody at 10 $\mu\text{g/ml}$ suppressed the T-IC properties of HCC cells, including tumorigenicity, self-renewal and chemoresistance. Given the metastatic role of CD47 in various cancer types (5, 11, 17), we found that the anti-CD47 antibody suppressed the migration and invasion abilities of HCC cells and that this effect was independent on the phagocytic role of CD47. In addition to its antiphagocytic functions, we previously reported that an anti-CD47 antibody suppressed CTSS/PAR2 signalling (5), which may suggest that the sensitization effect of the CD47 antibody to doxorubicin may be because of inhibitory signalling through CTSS/PAR2. In addition, CD47 was

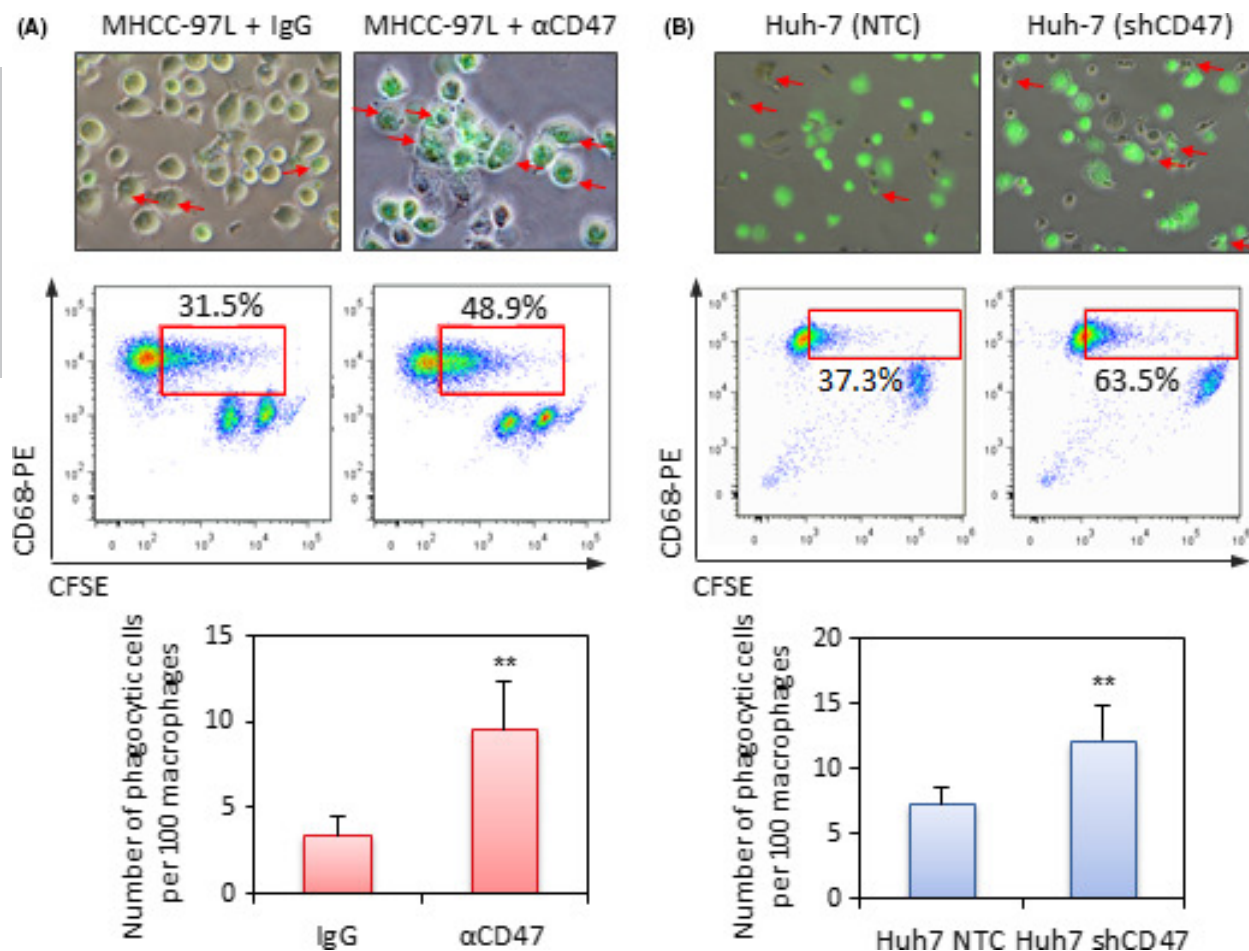


Fig. 4. Blockade of CD47 by the anti-CD47 antibody induced macrophage-induced phagocytosis. (A) Blockade of the CD47-SIRP α interaction enabled the phagocytosis of HCC cells (** $P < 0.010$, t test). Representative images of human peripheral blood (PB)-derived macrophages phagocytosing MHCC-97L cells following treatment with the anti-CD47 antibody (B6H12) and control IgG. Arrows point to phagocytosed HCC cells. Upon anti-CD47 treatment, the percentage of CFSE⁺CD68⁺ macrophages increased from 31.5% to 48.9%. (B) Knockdown of CD47 in Huh-7 cells significantly increased the number of phagocytosed cells (** $P < 0.010$, t test). Representative images of human peripheral blood (PB)-derived macrophages phagocytosing shCD47 and control cells. Arrows point to phagocytosed HCC cells. The percentages of CFSE⁺CD68⁺ macrophages were 63.5% and 37.3% for shCD47 and control (NTC) cells respectively. **12**

also previously reported by other groups to bind to thrombospondin-1 (TSP-1) (18). TSP-1 is known to be a protease, and as such, it may be able to affect many yet-unknown pathways that lead to sensitization to chemotherapy.

7 SIRP α is expressed on phagocytes and inhibits phagocytosis upon binding to CD47 (19). It is because of this function that CD47 was coined as a 'do-not-eat-me' signal. Willingham *et al.* (12) reported that human solid tumour cells express CD47 as a typical mechanism to evade phagocytosis. Using an anti-CD47 monoclonal antibody to block CD47 binding to SIRP α , the 'do-not-eat-me' signal is disrupted, and clearance of the solid tumour by phagocytosis occurs. To examine whether blockade of CD47 by an anti-CD47 antibody would induce macrophage-induced phagocytosis in HCC cells, we pretreated MHCC-97L

cells with either an anti-CD47 antibody or control IgG in the presence of human macrophages. Based on our phagocytosis assay, we found that MHCC-97L cells treated with the anti-CD47 antibody were efficiently phagocytosed by human macrophages. To further verify whether CD47 is crucial in this process, we repressed the expression of CD47 in Huh-7 cells and examined its effect on phagocytosis. Our data showed that knockdown of CD47 resulted in a significant increase in the number of phagocytosed HCC cells. This result is consistent with other report showing increase in macrophage-mediated phagocytosis upon knockdown of CD47 in melanoma (20). In summary, our results showed the inhibitory role of CD47 in macrophage-mediated phagocytosis and that its blockade by the anti-CD47 antibody induced phagocytosis of HCC cells.

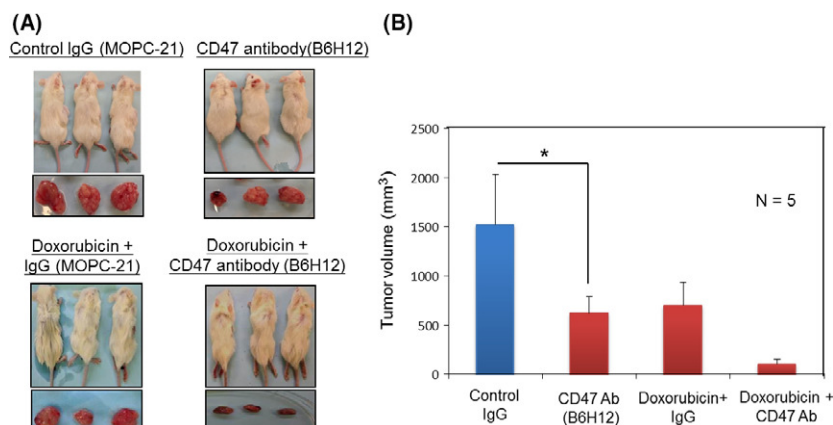


Fig. 5. Anti-CD47 antibody in combination with doxorubicin in an HCC PDX model. The *in vivo* therapeutic effect of the anti-CD47 antibody was examined in a NOD/SCID mouse model using patient-derived xenograft (PDX #1). The anti-CD47 antibody significantly reduced tumour size by approximately two-fold compared with the IgG control (* $P < 0.010$, *t* test), and its effect was more potent than doxorubicin plus IgG. Similarly, the combination of doxorubicin (Dox) and the anti-CD47 antibody exhibited a synergistic effect (~10.1-fold) on tumour suppression. Three representative mice in each group are shown.

Several studies have utilized xenograft mouse models to evaluate the anticancer effects of anti-CD47 antibodies and demonstrated a marked effect on tumour suppression over a wide range of cancers (9–12). Although targeting CD47 were found efficacious in several pre-clinical tumour models, combination strategies involving suppression of CD47-SIRP α pathway offered greater therapeutic potential. In NHL, combination of anti-CD47 antibody with anti-CD20 antibody rituximab led to synergistic elimination in mouse model of NHL (6). In addition, anti-SIRP α antibody was found to potentiate the effect of anti-Her2 antibody trastuzumab against breast cancer cells (21). In this study, we attempted to evaluate if there was a synergistic effect when anti-CD47 antibody in combination with chemotherapy. In *in vitro* experiments, we found that such synergistic effect was observed in both Huh-7 and MHCC-97L cells in response to cisplatin or doxorubicin, and it is not cell-type and drug specific. To further confirm this finding, we have examined the *in vivo* combined effect of doxorubicin with anti-CD47 antibody in NOD/SCID mice bearing PDX#1. The results from this experiment were encouraging, as doxorubicin and anti-CD47 antibody treatments alone were only able to produce a 2.17- to 2.4-fold suppression in tumour volume, whereas the combined treatment produced an astonishing 10.1-fold suppression. This observation suggests a synergistic effect of the combination treatment of doxorubicin and the anti-CD47 antibody. The therapeutic efficacy of doxorubicin is also evidently improved by combination therapy. Moreover, no visible toxic effects caused by the anti-CD47 antibody were observed.

In conclusion, we demonstrated that an anti-CD47 antibody suppresses the T-IC properties of HCC cells and efficiently induces macrophage-mediated phagocytosis. More importantly, the administration of an anti-CD47

antibody suppressed *in vivo* tumour growth and exerted a synergistic effect with doxorubicin treatment. Thus, targeting CD47 with an anti-CD47 antibody in combination with doxorubicin appears to be a promising novel therapeutic strategy for the treatment of HCC.

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Conflict of interest: The authors do not have any disclosures to report.

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Supporting information

Additional Supporting Information may be found at
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