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β1 integrin-mediated multicellular resistance in hepatocellular carcinoma through activation of the FAK/Akt pathway

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Abstract

Objective: To explore the role and mechanism of β1 integrin in the regulation of multicellular drug resistance in hepatocellular carcinoma (HCC).

Methods: This *in vitro* study used a liquid overlay technique to obtain multicellular spheroids of two human HCC cell lines, HepG2 and Bel-7402. The morphology of the spheroids was observed by optical and electron microscopy. The effects of exposure to 5-fluorouracil (5-FU) and cisplatin (CDDP) on cell proliferation and the induction of apoptosis were assessed in monolayer cells and multicellular spheroids. The levels of β1 integrin and the effects on the focal adhesion kinase (FAK)/protein kinase B (Akt) pathway were evaluated using Western blot analysis, immunofluorescence and flow cytometry. The role of β1 integrin was confirmed by using an inhibitory antibody.

Results: Cell proliferation inhibition and cell apoptosis induced by 5-FUl and CDDP were abrogated in multicellular spheroids compared with monolayer cells. There were high levels of β1 integrin in multicellular spheroids. β1 integrin inhibitory antibody prevented the formation of multicellular spheroids, coupled with a significant increase in proliferation inhibition and apoptosis induction. β1 integrin inhibitory antibody effectively suppressed activation of both FAK and Akt in multicellular spheroids.

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Conclusions: β1 integrin mediated multicellular drug resistance through the FAK/Akt pathway in HCC spheroids.

Keywords
β1 integrin, multicellular resistance, hepatocellular carcinoma, focal adhesion kinase, protein kinase B

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Introduction

Hepatocellular carcinoma (HCC) is the fourth most common liver malignancy and the second-leading cause of cancer-related death in China.[1] In 2015, the mortality rate of HCC was reported be 422.1 per 100 000.[1] Despite advances in HCC treatment, most patients with HCC die within 6–20 months after diagnosis and the 5-year survival rate is only 3%,[2] largely due to the aggressive biological characteristics of the tumours, which includes rapid tumour growth and their being highly refractory to chemotherapy. Therefore, there is considerable scientific interest in the molecular mechanisms responsible for chemotherapy resistance in HCC.

In recent years, employing *in vitro* 3D cultures, sometimes called tumour spheroids, has made a significant contribution to cancer drug resistance research.[3,4] Growing evidence from cancer research has revealed that tumour spheroids present a more physiologically relevant *in vitro* microenvironment compared with monolayer cell cultures due to the differences in cell morphology, cell organization, cell surface receptor expression, gene expression and cell signalling.[3,5–7] Previous studies have shown that tumour spheroids were generally more resistant to chemotherapy than their conventional 2D monolayer culture counterparts.[8–12] This novel concept, called multicellular resistance, emphasized the importance of utilizing tumour multicellular spheroids for the evaluation of anticancer drug and mechanistic studies.[13]

The integrin family, which contains 18 α-subunits and eight β-subunits, consists of transmembrane cell-surface adhesion receptors that mediate cell–cell and cell–matrix adhesion and interaction, which have been shown to regulate a multitude of biological behaviours, such as cell proliferation, polarity, invasion, angiogenesis and cancer therapy resistance.[14–16] β1 integrin, which can form heterodimers with many different α-subunits,[17] has been shown to bind to receptors that will support activation of downstream signalling cascade pathways regulating many physiological or pathological processes, particularly cell adhesion and communication, and tumour progression.[16] β1 integrin plays a critical role in recruiting focal adhesion kinase (FAK) and inducing its autophosphorylation on Y397.[16,18] Phosphorylation of FAK then results in activation of signalling molecules such as protein kinase B (Akt), paxillin, c-Src, and contributes to integrin signalling.[19–21] Studies have demonstrated the overexpression of β1 integrin and have investigated its role in the progression of HCC.[22,23] For example, β1 integrin expression is important to liver homeostasis because loss of β1 integrin impairs liver regeneration and HCC progression.[23,24] β1 integrin has also been implicated in participating in the process of hepatoma spheroid formation.[7] In addition, β1 integrin has

been linked to chemotherapy resistance in multiple cancer types in cell monolayer culture, including HCC, head and neck cancer, and breast cancer.[25–27] However, whether β1 integrin mediates chemotherapeutic drug resistance in HCC multicellular spheroids remains largely unclear.

The present study used a liquid overlay technique to obtain multicellular spheroids and compared the levels of β1 integrin in HCC monolayer cells and multicellular spheroids. The study also investigated the inhibition of proliferation and induction of apoptosis of HCC monolayer cells and multicellular spheroids in response to exposure to 5-fluorouracil (5-FU) and cisplatin (CDDP); and the role of β1 integrin in chemotherapy resistance in HCC multicellular spheroids.

Materials and methods

Monolayer cells and multicellular spheroid culture

HepG2 cells and Bel-7402 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium containing 10% fetal bovine serum in 5% CO_2 at 37°C as a monolayer culture. As previously described,[10–12] a liquid overlay technique was used to obtain tumour multicellular spheroids. Briefly, a cell suspension was seeded at 2 x 10^5 cells in each culture flask coated with 2% agarose (Sigma-Aldrich, St Louis, MO, USA) before cell plating. Tumour multicellular spheroids were obtained after incubation for 4 days and the formation process of multicellular spheroids was observed by means of optical microscopy (AE2000LED inverted microscope; Motic, Xiamen, China).

Ultrastructural observation

The monolayer cells and tumour multicellular spheroids were fixed in 2.5% glutaraldehyde for 2 h and then post-fixed on the plate with 1% OsO_4, and dehydrated by a graded series of ethanol. The cells were then covered with gold palladium and examined using scanning electron microscopy (SEM) (JSM-840; JEOL, Tokyo, Japan); or embedded in Epon812 epoxy resin and examined using transmission electron microscope (TEM) (H-600; Hitachi, Krefeld, Germany).

Cell proliferation inhibition assay

The cell proliferation inhibition effects of 5-FU and CDDP in monolayer cells and multicellular spheroids were determined using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described.[10–12] For each well, a total of 2500 cells were seeded as either monolayer cells or multicellular spheroids in 150 μl of tissue culture medium. Next, the cells were treated with 5-FU (Xudong Haipu Pharmaceutical Company, Shanghai, China) and CDDP (Qilu Pharmaceutical Company, Shandong, China) for 48 h at the indicated concentrations. Then, 20 μl of 5 mg/ml MTT (Sigma-Aldrich) was added. After 4 h of incubation at 37°C, 150 μl dimethyl sulfoxide (Sigma-Aldrich) was added to each well and the absorbance was measured at 492 nm on a POLARstar® Omega multifunction microplate reader (BMG LABTECH, Offenburg, Germany). All sample measurements were replicated five times. The cell proliferation inhibition was calculated by the formula: inhibitory ratio (%) = [1− (absorbance experiment well)/(absorbance untreated well)] × 100%.

Cell apoptosis assay

A total of 10^5 cells of monolayer cells or multicellular spheroids were incubated with the anticancer drug for 48 h. Then, the cells were digested with trypsin, harvested, suspended, stained with 50 μg/ml

propidium iodide (PI) (Sigma-Aldrich) and fluorescein isothiocyanate (FITC)-conjugated Annexin V antibody (Beyotime Institute of Biotechnology, Shanghai, China) in the dark for 15 min at room temperature. Cell apoptosis was assessed by flow cytometry using a FACSCalibur™ system (BD Biosciences, San Jose, CA, USA) and the data were analysed using BD CellQuest™ software (BD Biosciences). Apoptotic cells were defined as being annexin V^+/PI^-.

Caspase-3 activity

A total of 10^5 cells of monolayer cells or multicellular spheroids were incubated with the anticancer drug for 48 h. Then, the cells were digested with trypsin, harvested, and washed with ice-cold 0.01 M phosphate-buffered saline (PBS; pH 7.4), and resuspended in 50 μl of chilled cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) for 15 min on ice. The caspase-3 activity was measured using a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The colourimetric reaction was developed and measured at 405 nm on a POLARstar® Omega multifunction microplate reader (BMG LABTECH) in order to calculate the caspase-3 activity.

Immunofluorescence analysis

Monolayer cells and multicellular spheroids were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min and blocked with 5% bovine serum albumin (Sigma-Aldrich) for 1 h at room temperature. The cells were then stained with mouse antihuman β1 integrin monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight, followed by staining with FITC-conjugated rabbit antimouse immunoglobulin for 2 h (1:2000 dilution; Santa Cruz Biotechnology) and then 20 μg/ml PI for 15 min at room temperature. Immunofluorescence was visualized using a laser scanning spectral confocal microscope (TCS SP2; Leica, Solms, Germany).

Flow cytometry

Monolayer cells and multicellular spheroids were digested with trypsin, harvested, suspended in 0.01 M PBS (pH 7.4) at 10^6 cells/ml and incubated with mouse antihuman β1 integrin monoclonal antibody (1:50 dilution; Santa Cruz Biotechnology) at 37°C for 1 h. Cells were then incubated with FITC-conjugated rabbit antimouse immunoglobulin secondary antibody (1:100 dilution; Santa Cruz Biotechnology) for 30 min at room temperature. The levels of β1 integrin on monolayer cells and multicellular spheroids were assessed by flow cytometry using a FACSCalibur™ system (BD Biosciences) and the data were analysed using BD CellQuest™ software (BD Biosciences).

Western blot analysis

Monolayer cells and multicellular spheroids treated with or without anticancer drug for 48 h were lysed using cell lysis buffer (40 mmol/l Tris-HCl [pH 7.4], 10% glycerol, 50 mmol/l bisphosphoglyceric acid, 5 mmol/l ethylenebis(oxyethylenenitrilo)tetra-acetic acid, 2 mmol/l ethylenediaminetetra-acetic acid, 0.35 mmol/l vanadate, 10 mmol/l NaF, and 0.3% Triton X-100) containing protease inhibitors (Roche Diagnostics, Penzberg, Germany). Equivalent amounts of protein (50 μg) detected by the bicinchoninic acid assay were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to Immobilon® membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h at room

temperature and incubated overnight at 4°C with primary antibodies as follows: antihuman β1 integrin antibody (1:200 dilution), antihuman Akt1/2 antibody (1:500 dilution), antihuman FAK antibody (1:200 dilution), antihuman α1 integrin (1:200 dilution), antihuman α5 integrin 1 (1:300 dilution), and antihuman β-actin antibody (1:800 dilution) were purchased from Santa Cruz Biotechnology; and the antihuman phospho-Akt (Ser473) antibody (1:100 dilution) and antihuman phospho-FAK (Tyr397) antibody (1:100 dilution) were obtained from Cell Signaling Technology®. The membranes were washed three times after incubation with the primary antibody using Tris-buffered saline Tween-20 (TBST; pH 7.4; 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20). Blots were incubated with a rabbit antimouse immunoglobulin secondary antibody coupled to horseradish peroxidase (1:2500 dilution; Santa Cruz Biotechnology) for 30 min at temperature. The membranes were washed three times after incubation with the secondary antibody using TBST (pH 7.4). Protein bands were visualized using an enhanced chemiluminescence detection system (Luminata™ Western HRP Substrates; Millipore).

Inhibitory antibody assays

Multicellular spheroids were treated with anticancer drug combined with β1 integrin inhibitory antibody (0.2 mg/ml; clone AIIB2; Millipore) or 0.2 mg/ml isotype immunoglobulin G (IgG) antibody (Millipore) as control or FAK inhibitor (defactinib 1 μmol/l; MCE, Shanghai, China). Then, spheroid formation, proliferation inhibition, cell apoptosis and protein level experiments were assessed as described above.

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 13.0 (SPSS Inc., Chicago, IL, USA) for Windows®. All data are shown as mean ± SD. Variance analysis and Student's *t*-test were used to analyse the data. A P-value < 0.05 was considered statistically significant.

Results

To assess the differences in drug sensitivities between conventional 2D monolayer cell culture and 3D culture, this study used a liquid overlay technique[10–12] to obtain HCC tumour spheroids closely resembling the *in vivo* situation (Figure 1). The study tested various starting cell numbers per well (data not shown) and 2 x 10^5 cells formed spheroids during a 4-day growth period. The growth of HCC tumour spheroids was dynamically assessed using optical microscopy every day. In brief, small aggregations of cells were observed on day 1, which was followed by gradually more compact growth, until on day 4, larger multicellular spheroids had developed (Figure 1). HepG2 cells formed large, tightly round multicellular spheroids (Figure 1a), while Bel-7402 formed oval multicellular spheroids (Figure 1b).

Scanning EM and TEM were used to assess the ultrastructural morphology of multicellular spheroids. SEM showed that HCC multicellular spheroids were different sizes, with diameters ranging from 100 to 200 μm after 4 days; and their shapes were round, oval spheroid, and polyhedron or tightly packed. The multicellular spheroids from the two cell lines were different. For example, HepG2 multicellular spheroids were round (Figure 1c), while Bel-7402 multicellular spheroids were oval (Figure 1d). TEM showed abundant desmosome junctions in the multicellular spheroids, which are the ultrastructural basis of the interaction and communication between cancer cells. A lumen-like structure was formed between the tight junctions of the cells

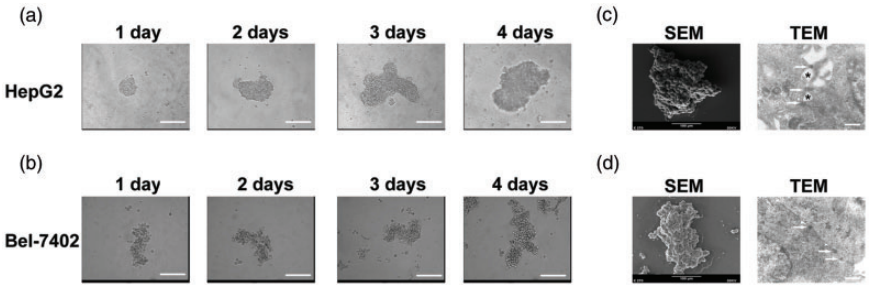

Figure 1. Multicellular spheroid formation and morphological characterization. Multicellular spheroid formation of HepG2 cells (a) and Bel-7402 (b), photographed from day 1 to day 4 by optical microscopy (scale bar 100 μm). Ultrastructural morphology of multicellular spheroids of HepG2 cells (c) and Bel-7402 (d) by scanning electron microscopy (SEM; scale bar 100 μm) and transmission electron microscopy (TEM; scale bar 10 000 μm). Arrows show desmosome junctions and asterisks show lumen-like structures.

in the HepG2 multicellular spheroids (Figure 1c), while the same organization was not observed in the Bel-7402 multicellular spheroids (Figure 1d).

Given the different ultrastructural morphology between monolayer cells and multicellular spheroids, the study then investigated whether there was resistance to chemotherapy in multicellular spheroids. The HepG2 and Bel-7402 multicellular spheroids were resistant to 5-FU and CDDP as determined by the MTT assay (Figure 2a and Figure 2b, respectively). For example, 5-FU inhibited cell proliferation of HepG2 monolayer cells by 33–81%, whereas the inhibitory ratios for the same concentrations of 5-FU were 18–36% in HepG2 multicellular spheroids (Figure 2a). Similar observations were made in the Bel-7402 cells (Figure 2b).

The study then determined whether cell apoptosis was induced by 5-FU and CDDP in monolayer cells and tumour spheroids using flow cytometry. 5-FU and CDDP induced cell apoptosis in monolayer cells and multicellular spheroids of HepG2 and Bel-7402 in a dose-dependent manner (Figure 2c and Figure 2d). However, multicellular spheroids of both HepG2 and Bel-7402 had significantly fewer apoptotic cells than monolayer cells ($P < 0.05$ for all comparisons), except for 5mg/l CDDP, which did not produce a significant difference between the monolayer cells and multicellular spheroids of Bel-7402 (Figure 2c and Figure 2d). The caspase-3 activity data also suggested that multicellular spheroids of both HepG2 and Bel-7402 demonstrated less apoptosis following exposure to 5-FU and CDDP (Figure 2e and Figure 2f).

β1 integrin is considered an essential player in mediating cell–cell and cell–matrix adhesion and interaction[14–16] and hepatoma spheroid formation.[7] Therefore, the study hypothesized that there would be high levels of β1 integrin in HCC multicellular spheroids. The identification and cellular localization of β1 integrin in monolayer cells and multicellular spheroids was investigated by immunofluorescence, flow cytometry and Western blot analysis (Figure 3). Immunofluorescence analysis revealed that, similar to monolayer cells, β1 integrin was predominantly located in the cell membrane in multicellular spheroids of HepG2 and Bel-7402 (Figure 3a). High levels of β1 integrin were observed in the close contacts between cancer cells, especially in multicellular spheroids (Figure 3a). The mean fluorescence

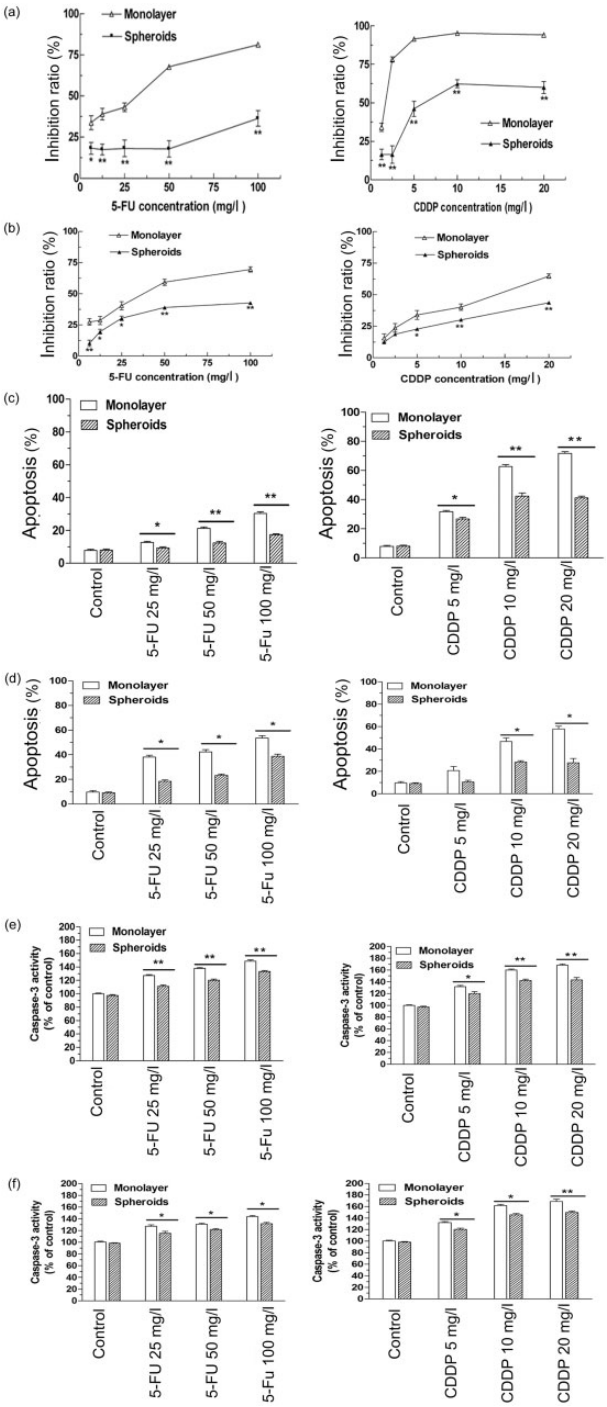

Figure 2. Cell proliferation inhibition and cell apoptosis. Cell proliferation inhibition after 48 h treatment with 5-fluorouracil (5-FU) and cisplatin (CDDP) in HepG2 (a) and Bel-7402 (b) cells. Cell apoptosis after 48 h treatment with 5-FU and CDDP in HepG2 (c) and Bel-7402 (d) cells. Caspase-3 activity after 48 h treatment with 5-FU and CDDP in HepG2 (e) and Bel-7402 (f) cells. Data presented as mean $\pm$ SD. $^{*}P < 0.05$, $^{**}P < 0.01$; Student's *t*-test.

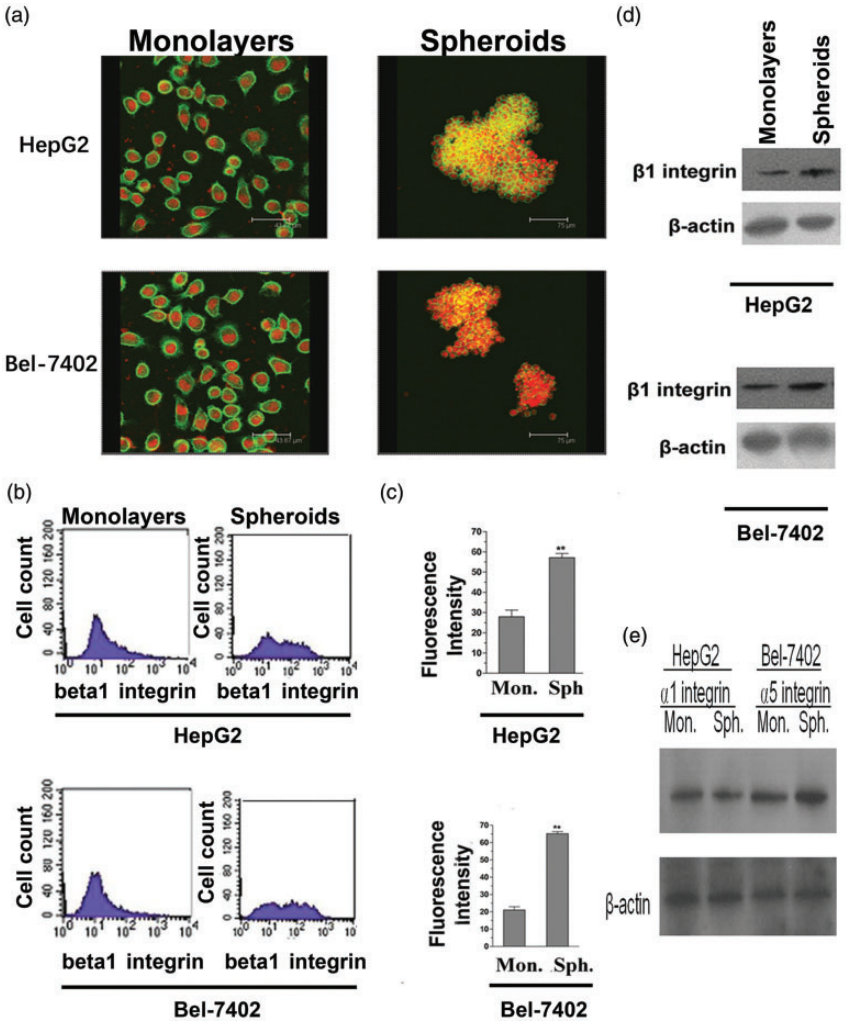

Figure 3. Identification of β1 integrin in monolayer cells and multicellular spheroids of HepG2 and Bel-7402 by immunofluorescence analysis (scale bar is 43.75 μm in monolayer cells; scale bar is 75 μm in multicellular spheroids; green fluorescence indicates β1 integrin and red fluorescence indicates the cell nucleus) (a). Flow cytometry (b), mean fluorescence intensity of β1 integrin labelling in monolayer cells and multicellular spheroids (c) and Western blot analysis (d). Preliminary experiments showing the levels of α1 integrin and α5 integrin in monolayer cells and tumour spheroids in hepatocellular carcinoma (e). β-actin was used as the loading control. Mon, monolayers; Sph, spheroids. Data presented as mean ± SD. $^{**}P < 0.01$; Student's *t*-test.

intensity of β1 integrin, which was detected by flow cytometry, significantly increased in multicellular spheroids compared with monolayer cells (Figure 3b and 3c) ($P < 0.01$ for both comparisons). For example, the expression of β1 integrin of HepG2 multicellular spheroids was two-fold higher than that of monolayer cells. Similarly, this value was three-fold higher in Bel-7402. Western blot analysis also

confirmed higher levels of β1 integrin in multicellular spheroids (Figure 3d). Preliminary experiments showed that there were no differences in the levels of α1 integrin and α5 integrin proteins between monolayer cells and tumour spheroids (Figure 3e), which is why β1 integrin was used in the main part of this study.

In view of increasing chemotherapy resistance and high levels of β1 integrin in multicellular spheroids, the study predicted that β1 integrin could mediate chemotherapy resistance of multicellular spheroids through its downstream signalling pathway. Therefore, the study then used a β1 integrin inhibitory antibody (clone AIIB2), which specifically binds to the β1 integrin extracellular domain and is considered as a function-blocking antibody,[28] to test this hypothesis. As β1 integrin regulates many aspects of tumour biology, including cell adhesion and cell communication, experiments were conducted to reveal the role of β1 integrin in HCC multicellular spheroid formation. Cell suspensions were seeded in each culture flask coated with 2% agarose before cell plating in the presence of β1 integrin inhibitory antibody or isotype IgG antibody as control. During 4 days of incubation, the inhibitory antibody continued to significantly reduce and retard the multicellular spheroid formation of HepG2 (Figure 4). The inhibitory antibody exhibited a stronger effect on preventing the multicellular spheroid formation of Bel-7402, with only loose cell masses not multicellular spheroids being formed after incubation for 4 days (Figure 4).

To investigate whether β1 integrin contributes to the increased chemotherapy resistance in multicellular spheroids of HepG2 and Bel-7402, the cells were incubated with anti-β1 integrin function-blocking antibody or isotype IgG antibody as control. The proliferation inhibition response was markedly enhanced when multicellular spheroids of HepG2 were

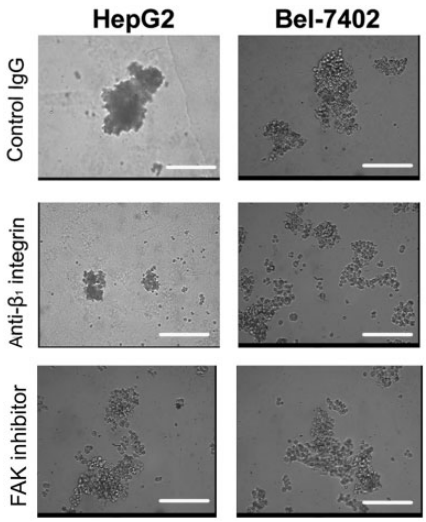

Figure 4. The role of β1 integrin in hepatocellular carcinoma multicellular spheroid formation. β1 integrin inhibitory antibody and focal adhesion kinase (FAK) inhibitor (defactinib) reduced and retarded multicellular spheroid formation of HepG2 and Bel-7402 (scale bar 100 μm).

treated with anticancer drug plus β1 integrin inhibitory antibody compared with control IgG (Figure 5a). The inhibitory antibody had a similar effect on the multicellular spheroids of Bel-7402 (Figure 5b). Cell apoptosis experiments were conducted to further confirm the above findings. The percentage of apoptotic cells was significantly increased when the multicellular spheroids of HepG2 and Bel-7402 were treated with anticancer drug plus β1 integrin inhibitory antibody compared with control IgG (Figure 5c and 5d) ($P < 0.05$ for all comparisons). Similarly, the caspase-3 activity was significantly enhanced when multicellular spheroids of HepG2 and Bel-7402 were treated with anticancer drug plus β1 integrin inhibitory antibody compared with control IgG (Figure 5e and Figure 5f) ($P < 0.05$ for all comparisons).

Since several studies have reported that β1 integrin exhibits many biological tumour

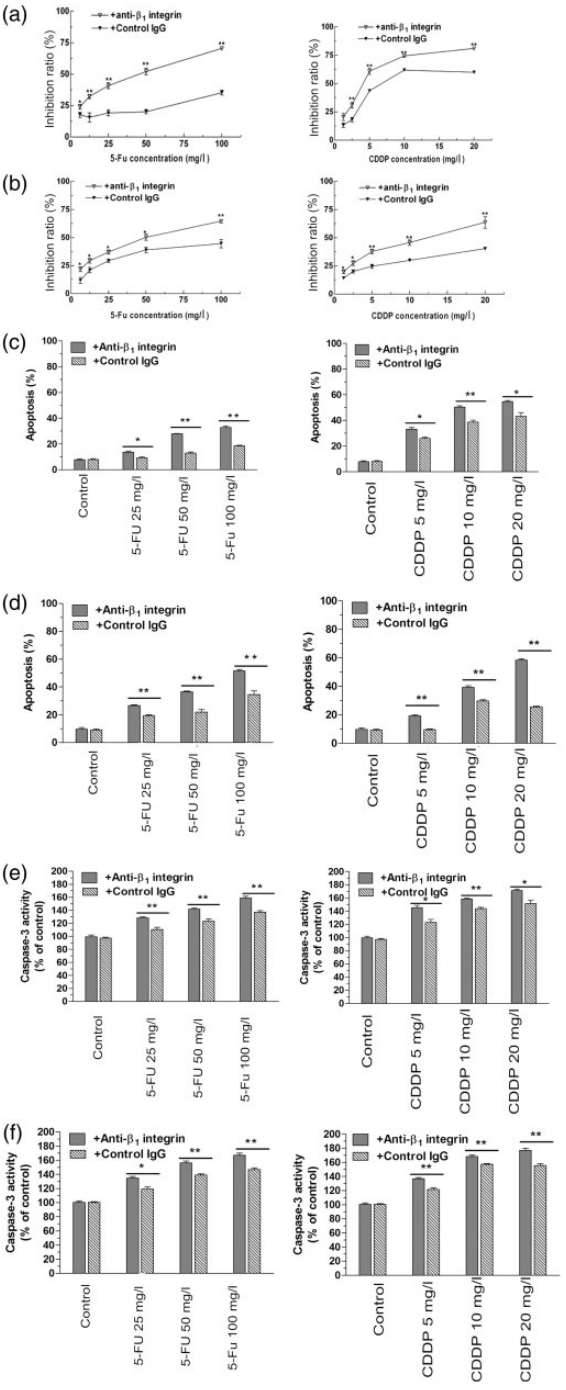

Figure 5. Investigation of β1 integrin-mediated resistance of multicellular spheroids. Addition of β1 integrin inhibitory antibody altered the inhibition of cell proliferation to 5-fluorouracil (5-FU) and cisplatin (CDDP) in multicellular spheroids of HepG2 (a) and Bel-7402 (b). Addition of β1 integrin inhibitory antibody altered the induction of cell apoptosis in response to 5-FU and CDDP in multicellular spheroids of HepG2 (c) and Bel-7402 (d); and caspase-3 activity to 5-FU and CDDP in multicellular spheroids of HepG2 (e) and Bel-7402 (f). IgG, immunoglobulin G. Data presented as mean ± SD. *$P < 0.05$, **$P < 0.01$; Student's *t*-test.

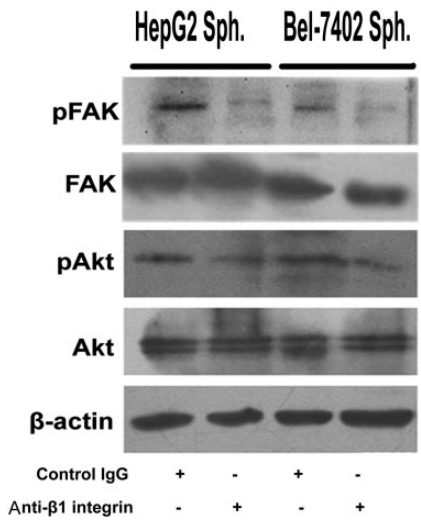

Figure 6. The role of β1 integrin in hepatocellular carcinoma multicellular spheroid formation. Western blot analysis showing β1 integrin inhibitory antibody decreased focal adhesion kinase (FAK) and protein kinase B (Akt) phosphorylation in multicellular spheroids of HepG2 and Bel-7402. β-actin was used as the loading control. IgG, immunoglobulin G; p, phosphorylated.

behaviours via effects on the FAK signalling pathway,[19,20] the study then examined the activation of FAK signalling and its downstream targets. Decreased FAK and Akt phosphorylation were observed in the multicellular spheroids of HepG2 and Bel-7402 treated by β1 integrin inhibitory antibody, but not by isotype IgG control (Figure 6). To explore the role of the FAK signalling pathway in multicellular resistance, the cells were treated with defactinib, which is a potent FAK phosphorylation inhibitor. The FAK inhibitor prevented multicellular spheroid formation of HepG2 and Bel-7402 (Figure 4). The percentage of apoptotic cells was significantly increased when the multicellular spheroids of HepG2 and Bel-7402 were treated with anticancer drug plus defactinib compared with control (Figure 7) ($P < 0.05$ for all comparisons).

Discussion

Hepatocellular carcinomas are aggressive cancers with a poor prognosis largely due to chemotherapy resistance.[2] Among the myriad of microenvironmental factors impacting on chemotherapy resistance, cell–cell adhesion and communication has recently been identified as a key determinant.[29,30] β1 integrin is a vital cell adhesion receptor with the ability to mediate cell–cell adhesion through activation of the FAK signalling pathway.[14,16] Previous studies have shown that there are high levels of β1 integrin in HCC tissues and that it is involved in tumour progression and drug resistance.[22,25] However, the role and mechanism of β1 integrin in HCC multicellular spheroid formation and multicellular spheroid drug resistance remain undetermined. This present study demonstrated that HepG2 and Bel-7402 cells could form multicellular spheroids with abundant desmosome junctions between individual cancer cells. The present study also observed that the inhibition of cell proliferation and the induction of cell apoptosis caused by 5-FU and CDDP treatment were abrogated in multicellular spheroids compared with monolayer cultures. In addition, there were high levels of β1 integrin in multicellular spheroids and β1 integrin mediated multicellular drug resistance through activation of the FAK/Akt signalling pathway.

The present study found that multicellular spheroids of HepG2 and Bel-7402 were able to resist proliferation inhibition and cell apoptosis induced by chemotherapy drugs (Figure 2), which confirmed that multicellular drug resistance exists in HCC. There is considerable evidence that 3D *in vitro* tumour cell cultures, such as multicellular spheroids, more accurately represent the complex *in vivo* microenvironment than conventional 2D monolayer cultures, particularly in terms of cell–cell contacts, cell–cell adhesion and gene expression

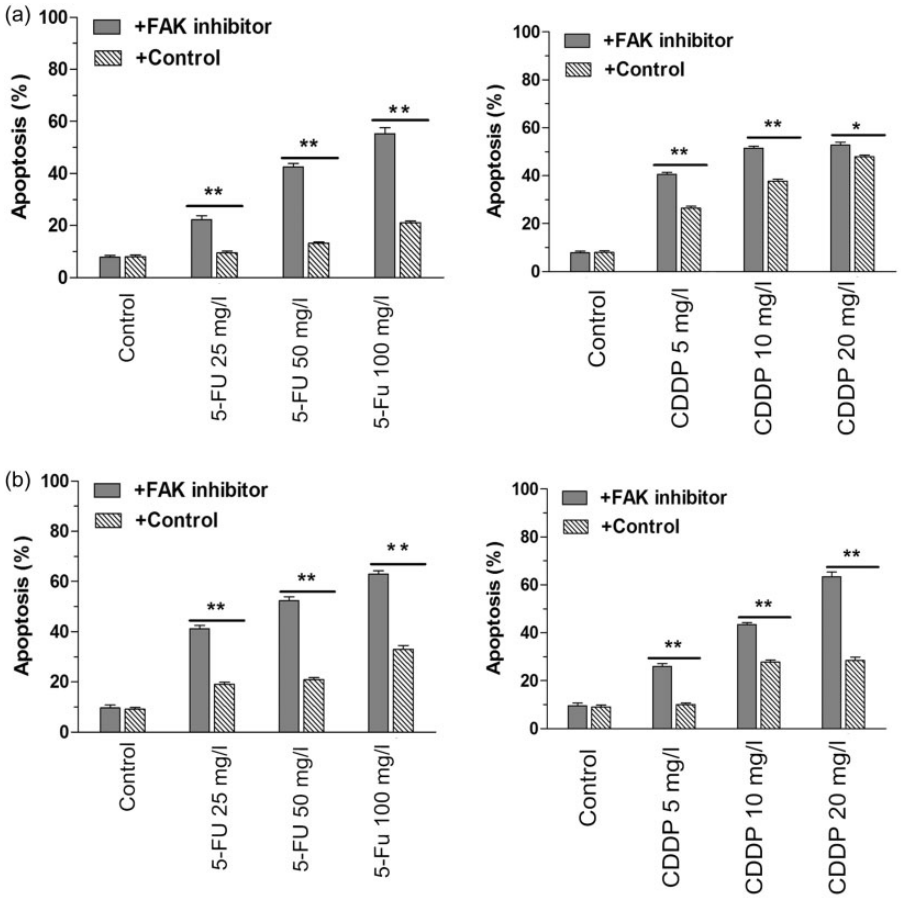

Figure 7. Investigation of the effect of the focal adhesion kinase (FAK) inhibitor defactinib on cell apoptosis. Addition of FAK inhibitor altered cell apoptosis in response to 5-fluorouracil (5-FU) and cisplatin (CDDP) in multicellular spheroids of HepG2 (a) and Bel-7402 (b). Data presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$; Student's *t*-test.

profiles.[31,32] This present study used a liquid overlay technique, which is a simple and convenient method,[10–12] to obtain HCC multicellular spheroids to perform these experiments. Although the morphology of the multicellular spheroids of HepG2 and Bel-7402 were different, both had abundant desmosome junctions between individual cancer cells (Figure 1), which suggests that closer contact and communication had developed between cancer cells compared with monolayer cultures. These close contacts between cancer cells may form the ultrastructural basis of chemotherapy resistance. It is not difficult to understand that if a high proportion of cancer cells are quiescent and the drugs have difficulty penetrating the 3D structure, then this might lead to multicellular drug resistance. Notably, lumen-like structures were observed in the multicellular spheroids of HepG2, which may be used to transport nutrients and waste in the same way as *in vivo* blood vessels. However, the same organization was

not observed in the multicellular spheroids of Bel-7402, which suggests cell type differences in the formation of multicellular spheroids.

It is known that the integrin family provides cells with adhesion and communication signals that are crucial for tumour initiation, angiogenesis, progression, metastasis, and chemotherapy resistance of solid tumours.[16] β1 integrin is one of the most important integrins, which is not only present in high levels in liver cancer tissue and a murine model,[22,23,33] but it also mediates cell adhesion that results in the activation of protein kinases that resist chemotherapy drugs.[14,16] This present study confirmed that high levels of β1 integrin are present in multicellular spheroids (Figure 3). Therefore, these findings suggest that it might be the close contact and adhesion between HCC cells and the associated high levels of β1 integrin that lead to multicellular drug resistance. The present study then used a β1 integrin inhibitory antibody to further validate these findings and the data clearly showed that in response to 4 days of β1 integrin inhibitory antibody treatment, both HepG2 and Bel-7402 could not form multicellular spheroids. There was also a significant decrease in the percentage of proliferating cells and a significant increase in the percentage of apoptotic cells (Figures 4 and 5). These current findings suggest that β1 integrin protected HCC cells from proliferation inhibition and apoptosis induced by 5-FU and CDDP.

It is widely understood that FAK is considered an essential player in integrin-mediated signalling.[21,29] β1 integrin recruits FAK and induces its autophosphorylation on Y397.[21] Phosphorylation of FAK also activates Akt through phosphorylating Akt at Ser473,[19] which transmits a survival signal.[34,35] In further elucidating the mechanism of action underlying β1 integrin, this present study found that β1 integrin inhibitory antibody effectively suppressed activation of both pFAK and pAkt in multicellular spheroids (Figure 6). Meanwhile, the FAK inhibitor defactinib also effectively prevented multicellular spheroid formation and increased cell apoptosis of the multicellular spheroids of HepG2 and Bel-7402 treated with anticancer drug plus defactinib (Figure 7). These current data suggest that β1 integrin mediates multicellular drug resistance of HCC cells through the FAK/Akt signalling pathway. However, future studies are required to confirm these results *in vivo*.

The results from this present study demonstrate that β1 integrin is not only present at high levels in multicellular spheroids of HCC cells, but it also protects HCC cells from proliferation inhibition and apoptosis induced by chemotherapy drugs through the FAK/Akt signalling pathway. These findings suggest that β1 integrin and its associated regulatory signalling pathways may be effective candidates for therapeutic intervention in the management of HCC.

Declaration of conflicting interests

The authors declare that there are no conflicts of interest.

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