Annexin A2 knockdown inhibits hepatoma cell growth and sensitizes hepatoma cells to 5-fluorouracil by regulating β-catenin and cyclin D1 expression

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common cancer types, and chemotherapy plays an important role in treatment of HCC. However, long-term treatment with chemotherapeutic drugs such as 5-fluorouracil (5-FU) often results in chemoresistance, and the underlying mechanisms remain unclear. In this study, we showed that the annexin A2 (ANXA2) protein is highly expressed in hepatoma cells compared to healthy cells. Knockdown of the *ANXA2* gene inhibited hepatoma cell growth, and the underlying mechanism may involve cell cycle inhibition through downregulation of β-catenin and cyclin D1. We also investigated the role of ANXA2 in chemotherapeutic treatment with 5-FU. 5-FU inhibited hepatoma cell growth, while *ANXA2* overexpression reduced, and knockdown enhanced, the effects of 5-FU on hepatoma cell growth. Furthermore, β-catenin and cyclin D1 were asscociated with the ANXA2-induced resistance. Taken together, our data suggest that the ANXA2 protein is a critical factor in HCC and that its downregulation can enhance chemotherapeutic treatment with 5-FU. ANXA2 may thus constitute a new therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer types worldwide and has an extremely poor prognosis (1,2). Certain risk factors, such as chronic hepatitis B and C virus infections, exposure to aflatoxin, non-alcoholic fatty liver disease and diabetes, contribute to HCC development. Although the etiology of HCC is well documented, the ability to treat HCC remains limited. Chemotherapy is commonly used in treatment of HCC. 5-Fluorouracil (5-FU) has been used to treat various cancer types, and is considered as a first-line anticancer drug for HCC chemotherapy worldwide. Unfortunately, its use is limited, since long-term treatment with this drug often results in chemoresistance, and the underlying mechanisms remain unclear (3,4). Therefore, it is necessary to further understand hepatocarcinogenesis and to explore new targets for the development of effective therapeutic strategies.

Annexins constitute a family of Ca^{2+}-dependent phospholipid-binding proteins with various membrane-related functions (5). At least 20 members of the annexin family have been described to date. Annexin A2 (ANXA2) is a multifunctional protein involved in numerous biological processes, including cellular transformation, cytoskeletal re-arrangement, and fibrinolysis. It is also implicated in a number of diseases, including inflammation, anti-phospholipid syndrome and cancer progression (6,7). ANXA2 is overexpressed in breast cancer, renal cell carcinoma, gastric carcinoma, glioma and multiple myeloma (8-12). By contrast, ANXA2 is downregulated in osteosarcoma and in head and neck squamous cell carcinoma (13,14). In HCC, ANXA2 expression has been found upregulated, and related to the histological grade of HCC (15-17). ANXA2 was also reported to promote the migration and invasion of hepatoma cells (18). However, the role of ANXA2 in hepatoma cell growth and chemotherapy remains unclear.

In this study, we analyzed the role of ANXA2 in hepatoma cell growth and found that *ANXA2* gene knockdown can inhibit hepatoma cell growth. We also investigated the mechanism mediating the antiproliferative effect of the *ANXA2* knockdown. In addition, we showed that *ANXA2* overexpression reduces, while knockdown enhances, the effects of 5-FU treatment on hepatoma cell growth. ANXA2 modulated the effects of 5-FU by regulating β-catenin and cyclin D1 expression. These data suggest that ANXA2 is a critical factor in HCC and plays an important role in chemotherapeutic treatment with 5-FU. ANXA2 may thus constitute a new therapeutic target for HCC.

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Materials and methods

Cell lines, plasmids and transfection. The human hepatocyte cell lines HepG2, Hep3B and normal human hepatocyte cell line L02 were purchased from the American Type Culture Collection (Manassas, VA, USA); in addition, the human hepatoma cell lines, SMMC-7721 and Huh7, were provided by Shanghai Cell Collection, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD, USA), as previously reported (19). A hairpin small interfering RNA (siRNA) was used to knock down the *ANXA2* gene in SMMC-7721 cells. The target sequences for *ANXA2* were: siANXA2a, 5'-GCGGGATGCTTTGAACATT-3', and siANXA2b, 5'-CGACGAGGACTCTCTCATT-3'. The siRNAs were obtained from Shanghai GenePharma Co., Ltd (Shanghai, China), and were inserted into the pSilencer 4.1-CMV-neo vector (Life Technologies) to generate the p-siANXA2a, p-siANXA2b and p-siNC knockdown plasmids. Negative pSilencer 4.1-CMV-neo vector (Life Technologies) that expresses a hairpin siRNA with limited homology to any known sequences in human, mouse, and rat genomes was used as the negative control (siNC). For the overexpression of *ANXA2* , total RNA was extracted from SMMC-7721 cells with the RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA, USA), following the manufacturer's instructions. Then, cDNA was synthesized from total RNA using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA), and the *ANXA2* gene was amplified from this cDNA using the PCR Master Mix (Promega, Madison, WI, USA) and the following cycling conditions: One cycle at 95˚C for 2 min followed by 30 cycles of 95˚C for 45 sec, 55˚C for 45 sec, and 72˚C for 90 sec, followed by an extension cycle of 72˚C for 5 min. The amplified cDNA was purified by QIAquick Gel Extraction Kit (Promega), and then introduced into the pcDNA3.1(-) expression vector (Life Technologies) to generate the *ANXA2* expression plasmid. All transfections were performed using Invitrogen™ Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Western blot analysis. Cells were lysed using SDS sample buffer [62.5 mM Tris-HCl, pH 6.8; 2% (wt/vol) SDS; 10% glycerol; 50 mM dithiothreitol, 0.1% (wt/vol) bromphenol blue] obtained from Sangon Biotech Co.,. Ltd. (Shanghai, China). Lysates were then separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham, Piscataway, NJ, USA). The membranes were blocked with 5% nonfat milk and then incubated with rabbit anti-human polyclonal antibodies targeting ANXA2, β-catenin, cyclin D1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), as indicated by the manufacturer. After washing in tris-buffered saline with Tween 20 (0.5% v/v), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies for 1 h, and visualized using the Enhanced Chemiluminescence Plus kit (Amersham). Band intensities were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). All the experiments were independently repeated at least three times.

Colony formation assay. Cells were plated on 6-well culture dishes in the presence of 500 μg/ml of G418 (Sigma-Aldrich, St. Louis, MO, USA) after treatment. The DMEM medium was replaced every three days with fresh medium containing G418. In certain experiments, the cells were also incubated with various concentrations of 5-FU, purchased from Sigma-Aldrich. Colonies were stained using crystal violet and counted 2 weeks following transfection under an inverted microscope (Olympus, Tokyo, Japan). All the experiments were performed in triplicate wells three times.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of the cells was assessed using an MTT-based Cell Growth Determination kit (Sigma-Aldrich). Cells at the logarithmic growth phase were plated in 96-well dishes in triplicate wells. Seventy-two hours following treatment, MTT (500 mg/ml) was added to the cells and cells were left to incubate for an additional 4 h. The absorbance of the formazan product was measured on an enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA, USA). Each assay was repeated three times.

Cell cycle analysis. Cells were harvested 48 h after treatment, and fixed in ice-cold 70% ethanol for 2 h. Invitrogen™ RNase A (1 mg/ml; Thermo Fisher Scientific) was added, and incubated with the cells at 37˚C for 30 min. Then, propidium iodide (50 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added, and the cells were incubated at 4˚C for 30 min away from light. The samples were immediately subjected to flow cytometry analysis (Navios instrument; Beckman Coulter, Miami, FL, USA). Cell cycle analysis data were analyzed using the MultiCycle for Windows software (Phoenix Flow Systems, San Diego, CA, USA). Experiments were repeated in triplicate. Average and standard deviation (SD) values were computed.

Statistical analysis. All experiments were carried out at least three times. Statistical analysis was conducted using the SPSS software (IBM, Armonk, NY, USA). Data were expressed as the means ± SD. The significance of the differences between groups was determined with Student's t-tests. $P<0.05$ was considered to indicate statistically significant differences.

Results

ANXA2 knockdown inhibits hepatoma cell growth. To study the role of ANXA2, we first studied its expression pattern in hepatoma cells by western blot. Compared to the level of ANXA2 in a the healthy hepatocyte line L02, the expression of ANXA2 was increased in the four hepatoma cell lines (Hep3B, HepG2, SMMC-7721 and Huh 7), with the highest level observed in the SMMC-7721 cell line (Fig. 1A). We next knocked down the *ANXA2* gene in the SMMC-7721 cell line. Western blot analysis showed that ANXA2 expression is markedly reduced in both knockdown cell lines (siANXA2a and siANXA2b), but not in the control cells, transfected with the p-siNC plasmid (Fig. 1B). We then examined the growth of these cell lines using an MTT assay. A reduction in viability was observed in both ANXA2-knockdown cell lines compared to the control cells (Fig. 1C). Moreover, *ANXA2* knockdown significantly reduced colony formation (Fig. 1D). These data

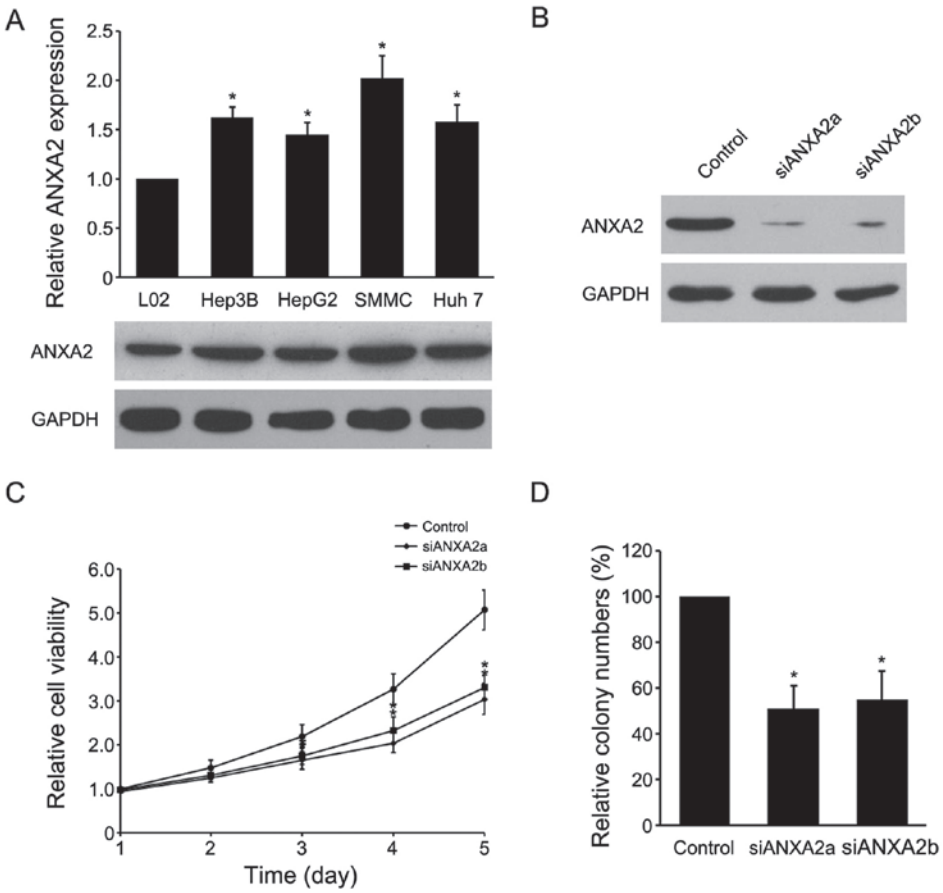

Figure 1. Increased expression of annexin A2 (ANXA2) in hepatoma cell lines and inhibition of hepatoma cell growth by the *ANXA2*-knockdown. (A) Western blot analysis (lower panel) and quantification of western blot (upper panel) of the ANXA2 protein in a hepatocyte cell line (L02) and in hepatoma cell lines (Hep3B, HepG2, SMMC-7721 and Huh 7). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The ANXA2 expression in L02 was set to 1.0. (B) The expression of ANXA2 was detected by western blot analysis in SMMC-7721 cells transfected with the small interfering RNA negative control (siNC; control in the figure), the siANXA2a, and the siANXA2b. *AXNA2* apears successfully silenced. (C) Cell viability of *ANXA2*-knockdown and control cells was determined by the MTT assay on days 1 to 5 after transfection. (D) Relative quantification of crystal violet-stained cells in the colony formation assay. The colony number in the control group was set to 100%. *P<0.05 compared to the control.

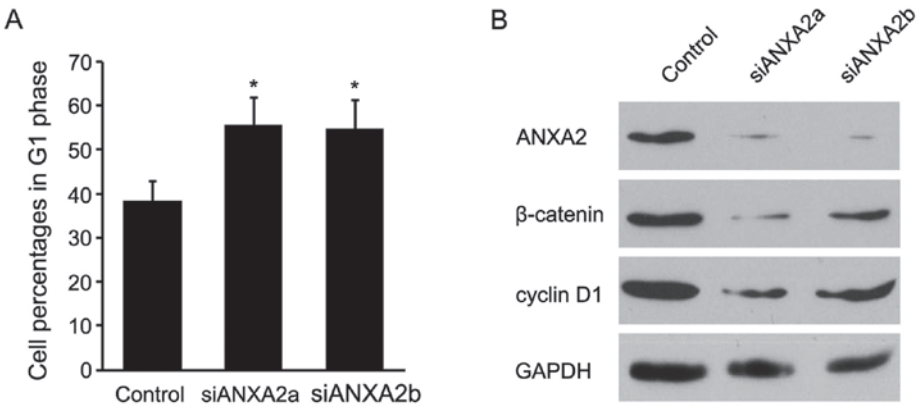

Figure 2. Annexin A2 gene (*ANXA2*) knockdown affects the cell cycle and the protein expression of β-catenin and cyclin D1. (A) Cell cycle progression was measured for cells transfected with the small interfering RNA negative control (siNC; control in the figure), siANXA2a, and siANXA2b. The percentage of cells at the G1 phase is shown. *P<0.05 compared to the control. (B) The expression of ANXA2, β-catenin, cyclin D1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected by western blot analysis in SMMC-7721 cells transfected with the siNC (control), siANXA2a, and siANXA2b.

suggested that knockdown of *ANXA2* inhibits hepatoma cell growth.

ANXA2 knockdown inhibits the cell cycle and the expression of β-catenin and cyclin D1. To investigate the mechanism mediating the antiproliferative effect of the *ANXA2* knockdown, we examined changes in the cell cycle by flow cytometry. This analysis showed that the *ANXA2* knockdown significantly increases the proportion of cells at the G1 phase (Fig. 2A), indicating that *ANXA2* knockdown may induce G1 to S phase

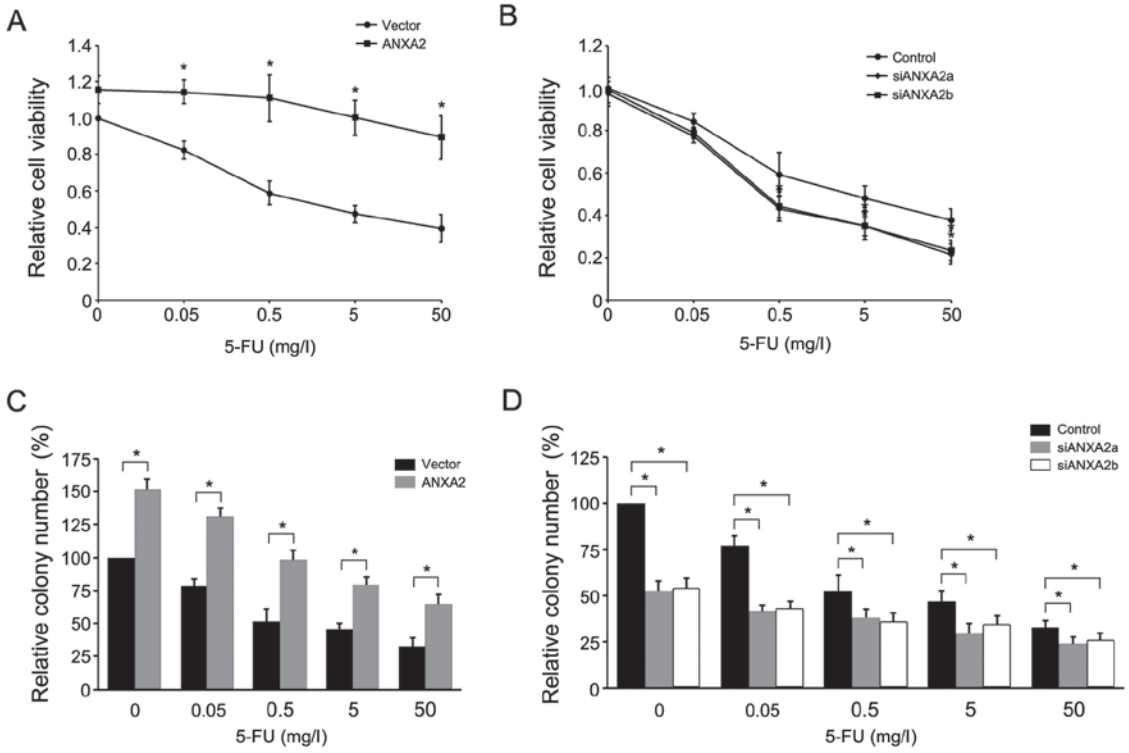

Figure 3. Annexin A2 gene (*ANXA2*) overexpression has antagonistic, while knockdown has synergistic effects on 5-fluorouracil (5-FU) treatment. (A) MTT assay and (C) colony formation assay showed that the anti-proliferative effects of 5-FU are less pronounced in the ANXA2-overexpressing cells compared to the cells transfected with the empty vector. (B) MTT assay and (D) colony formation assay showed that the anti-proliferative effects of 5-FU are more pronounced in the *ANXA2*-knockdown cells compared to the control cells (transfected with the small interfering RNA negative control). *P<0.05.

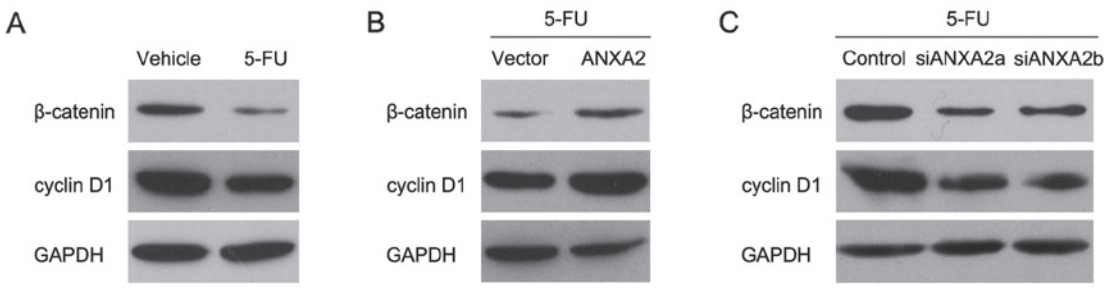

Figure 4. β-catenin and cyclin D1 are involved in annexin A2 (ANXA2)-induced resistance. The proteins of SMMC-7721 cells were extracted and subjected to western blot analysis to detect β-catenin and cyclin D1 expression under different conditions: (A) treatment with 50 mg/l of 5-fluorouracil (5-FU), (B) transfection with the *ANXA2* expression or the empty vector, in the presence of 5-FU (50 mg/l). (C) transfection with the small interfering RNA negative control (siNC; control in the figure), siANXA2a, and siANXA2b, in the presence of 5-FU (50 mg/l). Vehicle, cells treated with the vehicle solution Dulbecco's modified Eagle's medium.

arrest. Accordingly, we examined the expression of cell cycle regulators mediating the transition from the G1 to the S phase. Following *ANXA2* knockdown, the β-catenin and cyclin D1 levels were markedly reduced, especially in the siANXA2a line (Fig. 2B). These data suggested that *ANXA2* knockdown inhibits the cell cycle by regulating the expression of β-catenin and cyclin D1.

ANXA2 overexpression exerts antagonistic, while knockdown exerts synergistic effects with 5-FU. To evaluate the relationship between ANXA2 and 5-FU, the effect of 5-FU treatment on *ANXA2*-overexpressing and *ANXA2*-knockdown hepatoma cells was examined. 5-FU decreased the viability of hepatoma cells, similarly to previous studies (20,21). However, the viability of *ANXA2*-overexpressing hepatoma cells was not decreased by 5-FU treatment, indicating that *ANXA2* overexpression antagonizes 5-FU (Fig. 3A). On the other hand, hepatoma cells where *ANXA2* was silenced showed reduced viability upon 5-FU treatment, indicating that *ANXA2* knockdown and 5-FU act synergistically (Fig. 3B). Similar antagonistic and synergistic effects were also observed in the colony formation assay (Fig. 3C and D). These data demonstrated that *ANXA2* overexpression reduces, while knockdown enhances the inhibitory effects of 5-FU on hepatoma cell growth.

ANXA2 modulates the effects of 5-FU by regulating β-catenin and cyclin D1 expression. Since *ANXA2* expression modulated the effects of 5-FU, we investigated the underlying molecular mechanism. Upon treatment with 5-FU, the

hepatoma cells showed decreased expression of β-catenin and cyclin D1 (Fig. 4A). Given the reported roles of β-catenin and cyclin D1 in the cell cycle, these results indicated that 5-FU may inhibit hepatoma cell growth via downregulation of β-catenin and cyclin D1. Furthermore, we investigated the role of ANXA2 in the regulation of the two cell cycle-related proteins. Our data showed that *ANXA2* overexpression induces, while *ANXA2* knockdown decreases the expression of β-catenin and cyclin D1 under 5-FU treatment (Fig. 4B and C). Taken together, these data indicated that the ANXA2 protein modulates the effects of 5-FU by regulating β-catenin and cyclin D1 expression.

Discussion

ANXA2 is a multifunctional protein involved in numerous biological processes and diseases. It may act as a tumor suppressor or as an oncogene depending on the type of cancer. The expression of ANXA2 in HCC is upregulated and relates to the histological grade of HCC, which suggests that ANXA2 is a good diagnostic marker (15-17). Moreover, ANXA2 was shown to regulate HCC progression by promoting the migration and invasion of hepatoma cells (18). However, the role of ANXA2 in hepatoma cell growth and the underlying molecular mechanisms remain unclear. In this study, we showed that *ANXA2* knockdown decreases the proliferative ability of hepatoma cells, which indicates that ANXA2 plays a critical role in hepatoma cell growth. Accordingly, we examined the effects of ANXA2 on the cell cycle and found that the *ANXA2* knockdown induces G1 to S phase arrest. These data suggested that ANXA2 may be necessary for the transition from G1 to the S phase and for cell proliferation in HCC.

To investigate the mechanism by which ANXA2 regulates the cell cycle, we examined the expression of two cell cycle regulators controlling the transition from the G1 to the S phase, β-catenin and cyclin D1. Both proteins were found downregulated in the *ANXA2*-knockdown lines. The cellular level of β-catenin is mostly controlled by its ubiquitinylation and proteosomal degradation. Abnormal stabilized β-catenin accumulates and translocates into the nucleus to activate its target genes. Mutations and overexpression of β-catenin have been observed in HCC (22,23). Hepatocytes where β-catenin shows abnormal nuclear translocation also display abnormal cellular proliferation (24). β-catenin has been suggested as a promising target for HCC therapy in the clinic (25). Cyclin D1 is one of the key regulatory proteins controlling the transition from the G1 to the S phase. Amplification and overexpression of cyclin D1 was previously reported in HCC (26). In addition, knockdown of cyclin D1 interfered with hepatoma cell growth (27). Given that deregulation of cell cycle progression is a common event in human hepatocarcinogenesis, and that both β-catenin and cyclin D1 play carcinogenic roles in HCC, our data suggest that *ANXA2* knockdown inhibits cell cycle progression by downregulation of β-catenin and cyclin D1.

HCC has an extremely poor prognosis. Hepatectomy and liver transplantation are the most effective curative procedures for HCC patients. However, only ~10-15% of newly detected cases of HCC are amenable to surgical resection (28). Chemotherapy is one of the adjuvant treatment options available, and 5-FU is an important chemotherapeutic agent for HCC. However, the tumors may develop drug resistance after several cycles of 5-FU-based chemotherapy, through yet unclear mechanisms (3,4). ANXA2 was reported to be upregulated in a 5-FU-resistant cell line (29), however, its role in chemoresistance remains unclear. We found that *ANXA2* gene overexpression reduces the effects of 5-FU on hepatoma cell growth, indicating that this gene may play a role in chemoresistance. On the other hand, *ANXA2* knockdown sensitized hepatoma cells to 5-FU, which suggests that the ANXA2 protein could be used as a target to reduce chemoresistance during chemotherapy. We further investigated how ANXA2 functions in chemoresistance. The protein levels of β-catenin and cyclin D1, regulated by ANXA2, were also affected by 5-FU treatment. 5-FU decreased β-catenin and cyclin D1 expression. Moreover, activation of β-catenin and cyclin D1 was reported to induce chemoresistance to 5-FU (30). These data overall indicate that β-catenin and cyclin D1 play critical roles in 5-FU-related chemoresistance. Since ANXA2 regulates 5-FU-mediated expression of β-catenin and cyclin D1, we conclude that *ANXA2* knockdown sensitizes hepatoma cells to 5-FU by regulating β-catenin and cyclin D1 expression.

In conclusion, our study suggested that ANXA2 is a critical factor in HCC and plays an important role in chemotherapy when combined with 5-FU. ANXA2 may be a new therapeutic target for HCC.

Acknowledgements

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