Original Article

ANXA10 plays a suppressor role by regulating RhoA-ROCK signaling pathway in hepatocellular cancer

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Abstract: Object: This study was designed to investigate the role of ANXA10 in hepatocellular cancer. Methods: Lentiviral vector was used to increase the expression of ANXA10 in tumor cells. The expression of ANXA10 was confirmed by RT-qPCR and western blot method. CCK-8 kit was used to determine cell proliferation while cell invasiveness was detected with transwell assay. The cell apoptosis rate was determined with AnnexinV-FITC dye, and the in vivo effect of ANXA10 was evaluated in mice with xenograft tumor. Then Gene Expression Array was carried out to analyze the signaling pathway of ANXA10. Results: The level of ANXA10 was increased significantly in Huh-7 and SMMC-7721 cells. Cell proliferation was significantly inhibited after ANXA10 overexpression, and the invasiveness of SMMC-7721 cells was also inhibited. In contrast, ANXA10 overexpression induced cell apoptosis rate in both SMMC-7721 and Huh-7 cells. In vivo, ANXA10 overexpression also notably suppressed xenograft tumor growth. In addition, ANXA10 overexpression decreased expression of RhoA, Jun, MDM2 and ROCK. Also RhoA could rescue the adverse effects of ANXA10 overexpression on cell proliferation. Conclusion: ANXA10 plays a suppressor role in hepatocellular cancer and regulates RhoA-ROCK signaling pathway. This study gives us new clues to treat patients with hepatocellular cancer.

Keywords: ANXA10, cell apoptosis, RhoA, Jun, hepatocellular cancer

Introduction

The Annexin (ANXA) family is a multiple gene family consisting of 13 calcium and phospholipid-binding proteins [1, 2]. The 13 ANXAs are characterized by their structure of four or eight repeats of a 70-amino-acid motif and a variable N-terminal. Functional analysis proves the important role of the 13 ANXAs in multiple physiological activities such as immunosuppression, cell differentiation, proliferation, membrane transport, and calcium signaling [1-4]. Meanwhile, numerous studies demonstrate that abnormal expression, or subcellular localization of the 13 ANXAs affect the carcinogenesis of tumors by regulating cell proliferation, invasion or metastasis, apoptosis, and drug resistance [5]. For example, ANXA1 was associated with metastasis in breast cancer [6]. ANXA6 played a suppressor role in squamous cell carcinoma [7]. ANXA4 contributed to the resistance to paclitaxel in lung cancer cells [8]. ANXA10, mapped to chromosome 4q33, and has different functions in different cancers. For example, in pancreatic adenocarcinoma, ANXA10 expression promoted the progression of tumors [9, 10]. In oral cancer, ANXA10 activated the ERK/MAPK signaling pathway and accelerated the G1/S transition [11]. In colorectal cancer, ANXA10 exhibited an oncogene role [12]. However, in bladder, prostate, and hepatocellular cancer, ANXA10 may play a suppressor role in the development and progression of cancer [13-17]. Using hepatocellular cancer as an example, overexpression of ANXA10 in HepG2 cells inhibited proliferation and induced apoptosis, while weak ANXA10 expression was associated with poor prognosis in patients [16, 17]. Previous studies have suggested the suppressor role of ANXA10 in hepatocellular cancer, but more proof is necessary to confirm this, and the molecular mechanism of ANXA10 in hepatocellular cancer remains unclear.

Hepatocellular cancer (HCC) is a commonly seen malignant cancer and causes about 30
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thousand deaths each year in US [18]. According to reports by Siegel, the prognosis for HCC patients from 2007 to 2013 in US was disappointing. In brief, the estimated five-year survival rate for patients with localized HCC is 31%. It was 11% for those with regional HCC and only 3% for distant stage patients. What’s worse is the stage distribution of HCC patients. The patients diagnosed with distant stage account for about 18% of total HCC patients and 27% with regional stage. The patients with localized HCC account for 43%. In another word, the clinical outcome for HCC patients might be the worst of all top ten cancers. This could be partially attributed to the heterogeneity of HCC. HCC is a heterogeneous disease and next-generation sequencing technology has disclosed that a group of genes are responsible for the formation and progression of HCC [19-21]. However, the dominant gene is different in a particular cohort of patients. The disclosure of the dominant gene might contribute to the best therapy for cancer patients. The antibody drugs targeting a specific antigen in cancer have made it important to elucidate the dominant gene in HCC as well as the molecular mechanism. For example, avastin is a specific target drug with success in cancer therapy [22].

In this study, we explore the function of ANXA10 in the development and progression of HCC, where we overexpressed ANXA10 in two cell lines. Then we found that ANXA10 overexpression inhibited cell proliferation, migration and induced cell apoptosis in vitro. In vivo, ANXA10 also suppressed xenograft tumor growth potentiality. By using gene expression array and IPA analysis, we demonstrated that ANXA10 is involved in the regulation of the protein ubiquitination pathway, RhoA signaling, and TNFR1 signaling pathway. By using gene expression array and IPA analysis, we demonstrated that ANXA10 is involved in the regulation of the protein ubiquitination pathway, RhoA signaling, and TNFR1 signaling pathway. Western blot analysis proved that ANXA10 mainly regulated RhoA-ROCK signaling in HCC.

Materials and methods

Cell culture

Human hepatocellular carcinoma cell lines SMMC-7721 and Huh-7 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (ExCell Bio, Shanghai, China) with 10% fetal bovine serum (Gibco, MD, USA) in a humidified atmosphere with 5% CO₂ at 37°C.

Preparation of lentivirus vector expressing ANXA10 and infection of cells

The code sequence for the ANXA10 gene was synthesized and transferred into a lentivirus expression vector pCDH-GFP, which could be observed under a fluorescence microscope. Then HEK293T cells were used to produce the lentivirus particle. Briefly, HEK293T cells were co-transfected with 10 µg of pCDH-ANXA10/GFP or pCDH-GFP, 8 µg of psPAX, 4 µg of pMD2.G. After culture for 24 h, the supernatant was replaced with fresh medium and cultured for 72 h. The lentivirus particles were collected, and stored at -80°C.

Tumor cells including SMMC-7721 and Huh-7 were cultured and infected with lentivirus particle at approximately 80% of confluence for 6 h. After infection for 48 h, cells were observed under a fluorescence microscope and collected for RT-qPCR assay or western blot analysis.

RNA extraction and Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from cell lines with TaKaRa Minibest universal RNA Extraction kit (Takara, Dalian, China) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA with iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). RT-qPCR was carried out on ABI7000 system (Applied Biosystems, CA, USA) using SYBR. β-actin was the internal control. The relative amount of mRNA was evaluated with the 2-ΔΔCt method normalized to β-actin.

Western blot assay

A total of 15 µg of protein was separated on a 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to a PVDF membrane (Sangon Biotech, Shanghai, China). The PVDF membrane was incubated with primary antibody against ANXA10 (ab213665, 1:300, Abcam, NY, USA), RhoA (ab54835, 1:250, Abcam, NY, USA), ROCK (ab219587, 1:400, Abcam, NY, USA), Jun (ab119944, 1:200, Abcam, NY, USA), and MDM2 (ab38618, 1:200, Abcam, NY, USA) overnight at 4°C. After being washed with TBS, the membrane was incubated with secondary antibody at 25°C for 2 h. Then the protein was detected with enhanced chemiluminescence kit (Sangon Biotech, Shanghai, China). Tubulin was used as the internal control.
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**Proliferation assay (CCK-8)**

Cells were seeded into a 96-well plate at $5 \times 10^3$ cells/well after being treated with lentivirus carrying ANXA10. After being seeded for a consecutive 72 h, 10 µL of CCK-8 reagent was added and cells were cultured for another 4 h in the dark. Then the absorbance value at 450 nm wavelength was determined on a microplate reader. All experiments were repeated at least three times.

**Transwell assay**

Transwell plates with 8-µm-pore size membranes were treated with Matrigel matrix followed by inoculating with tumor cells. About $1 \times 10^4$ cells were seeded into the upper room of the chambers. DMEM with 20% fetal bovine serum was added into the lower chambers as the chemoattractant. After 48 h, cells on the upper side of the membrane were removed and cells in the lower side of the membrane were stained with 0.1% crystal violet for 15 min. Then stained cells were observed under a microscope.

**Apoptosis assay**

A total of $1 \times 10^5$ cells were washed with cold PBS and treated with Annexin V-FITC Apoptosis Kit (Beyotime, Shanghai, China). Briefly, 5 µL of FITC-Annexin V and 10 µL of propidium iodine were added into 100 µL of binding buffer and cells were treated with the mixed solution for 1 h in the dark at 25°C. Then the cells were analyzed with fluorescence-activated cell sorting (Beckman-Coulter, CA, USA) and the apoptotic rate was calculated.

**In vivo growth assay**

A total of 20 female BALB/c nu mice (6-7 weeks old, 20-25 g) were bought from Slac Laboratory (Shanghai, China) and were grouped as: ANXA10-oe and control. Then a total of $1 \times 10^7$ Huh-7 cancer cells treated with ANXA10-oe or control lentivirus particle were seeded into the right flank of the mice. The growth of the tumors was observed consecutively for 35 days. The volume of the tumor body was detected twice a week and calculated with the equation: $V=\frac{L \times W^2}{2}$, where $V$ is tumor volume,
MiRNA signature predicts survival rate of breast cancer.

L is length, and W is width of the tumor sphere. At the 35th day, all mice were anesthetized according to the guidelines approved by the Animal Research Ethics Committee of the First Affiliated Hospital of Nanchang University.

Statistical analysis

The data in this study were displayed as mean ± SD and all experiments were repeated at least three times. The statistical analysis was completed with software SPSS 11.0 (SPSS Inc, IL, USA). The difference between two groups was tested by Student’s t test and the difference between multiple groups was analyzed by One-Way ANOVA method followed by Turkey’s hoc test. P-value <0.05 was considered as a significant difference.

Results

ANXA10 was successfully overexpressed in hepatocellular cancer cells

To investigate the function of ANXA10 in hepatocellular cancer, ANXA10 was subcloned into the lentivirus expression vector pCDHGFP and the lentivirus particle (lent-ANXA10) was prepared in HEK293T cells. Then Huh-7 and SMMC-7721 cells were infected with lent-ANXA10 or control lentivirus lent-GFP, respectively. After infection for 48 h, GFP expression was observed under a fluorescence microscope. As shown in Figure 1A, the distribution of GFP was nearly 100%, which suggested the infection efficiency of lent-ANXA10 or lent-GFP was successful. In accordance, RT-qPCR assay demonstrated that ANXA10 expression increased greatly in both cell lines compared to the control after infection with lentivirus for 48 h (Figure 1B, 1C). Therefore, ANXA10 was successfully up-regulated in both Huh-7 and SMMC-7721 cells.

ANXA10 inhibited proliferation in hepatocellular cancer cells

ANXA10 is reported to inhibit cell proliferation in bladder cancer. In this study, we designed a CCK-8 assay to determine the effect of ANXA10 on the proliferation of hepatocellular cancer cells SMMC-7721 and Huh-7 cells. As shown in Figure 2A, the proliferation of Huh-7 cells was inhibited significantly at 72 h after lent-ANXA10 treatment. The cell number was decreased by about 2.5 fold. The proliferation of SMMC-7721 cells was also remarkably suppressed by lent-ANXA10 (Figure 2B). The cell number was decreased by about 1.9 fold after ANXA10 overexpression. So ANXA10 has shown it inhibits cell proliferation in hepatocellular cancer.

ANXA10 induced cell apoptosis in hepatocellular cancer

Anti-apoptosis is common in tumor cells and the majority of chemotherapeutic drugs killed tumor cells by inducing cell apoptosis. Here, by FACs analysis, we demonstrated that ANXA10 overexpression did enhance the cell apoptosis rate in hepatocellular cancer cell lines. As shown in Figure 3A, the apoptosis rate in lent-ANXA10-treated Huh-7 cells was nearly 3 folds of that in control. The apoptosis rate of SMMC-7721 cells was also increased by about 2 folds after ANXA10 overexpression (Figure 3C, 3D). We was concluded that ANXA10 overexpression induced cell apoptosis in hepatocellular cancer.
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Tumor metastasis is common in late stage patients. Potent migration ability or aggressiveness often predicts the metastasis in nearly all kinds of tumors. In this study, we designed a transwell assay experiment and demonstrated that ANXA10 overexpression suppressed the invasive ability of SMMC-7721 cells. As shown, the cell number transferred through the matrigel matrix was significantly less in ANXA10-overexpressed group compared to the control (Figure 4A, 4B). This data further supported that ANXA10 may play a suppressor role in hepatocellular cancer.

ANXA10 inhibited xenograft tumor growth in vivo

To explore the in vivo role of ANXA10, SMMC-7721 cells over-expressing ANXA10 were inoculated into nude mice subcutaneously. Then tumor growth was monitored and recorded at designed time points. As shown in Figure 5A, ANXA10 overexpression inhibited tumor growth significantly at day 21 after inoculation compared with the control group. This difference was further enlarged at the end point. At last, the weight of the tumor body was also decreased in ANXA10 overexpressing mice (0.83 g versus 0.11 g). ANXA10 reduced tumor weight by about 87% (Figure 5B). Therefore, ANXA10 overexpression suppressed xenograft tumor growth in vivo.

ANXA10 regulated RhoA-ROCK signaling pathway

To investigate the molecular mechanism of ANXA10 in hepatocellular cancer, gene expression array was used to dig out the downstream genes regulated by ANXA10. Then the data was analyzed by IPA database. A total of 905 genes were upregulated by ANXA10 expression and genes were 1016 were downregulated (Figure 6A). By enrichment analysis, we found that RhoA signaling was the one with most significant relevance to ANXA10 (Figure 6B). Then by western blot assay, we demonstrated that the expres-
sion of RhoA, ROCK, Jun, and MDM2 was significantly downregulated after ANXA10 overexpression in SMMC-7721 cells (Figure 6C). When RhoA was increased in SMMC-7721 cells, cell proliferation could be rescued even though ANXA10 was overexpressed (Figure 6D). Jun was also upregulated in RhoA expressed-SMMC-7721 cells. As a result, we deduced that ANXA10 might regulate RhoA-ROCK signaling pathway in hepatocellular cancer.

Discussion

Hepatocellular cancer is one of the ten leading causes of cancer-related deaths. A total of 30 thousand patients die of HCC each year and the prognosis is the worst of all the top ten cancers [18]. The current effective therapeutics for patients with HCC include surgical removal, chemotherapeutic drugs, radiotherapy and transplantation. A large cohort of HCC patients benefit very little from these therapies. One of the major reasons is heterogeneity of HCC, which is also a common phenomenon in all cancers [19]. The next-generation sequencing technology has disclosed that cancer is a result of accumulated gene mutations or alterations in the genome and the critical gene is often variable in different cohorts of patients [19-21]. So it is important to unveil the mask of HCC, and mining out new critical genes is a promising way to improve the prognosis of HCC patients.

The ANXA family is a multiple gene family and its 13 members play important roles in a series of physiological processes [1-4]. In past years, the 13 members have been reported to be involved in the development or progression of cancers [5]. ANXA10, as a distinct member in the ANXA family, has an unusual single codon deletion and loses its two main type II calcium-binding sites [1, 23]. ANXA10 is located at chromosome 4q33. It was reported that chromosome 4q is a common region with high frequent allelic loss in HCC [24]. In fact, ANXA10 was shown to promote tumorigenesis in gastric, pancreatic, colorectal and oral cancer, which suggested ANXA10 as an oncogene [9-12]. In other studies, low expression of ANXA10 was associated with progression of bladder cancer, and ANXA10 inhibited carcinogenesis in prostate cancer [13, 15]. In hepatocellular cancer, ANXA10 was shown to inhibit proliferation and induce apoptosis in HepG2 cells [16]. These studies indicated that ANXA10 might be a suppressor in HCC. In this study, we confirmed the function of ANXA10 in SMMC-7721 and Huh-7 cells. As shown above, ANXA10 can inhibit proliferation and invasiveness of the two cell lines, but increased cell apoptotic rate reversely. In vivo, ANXA10 also suppressed the growth of tumor cells. The suppressor role of ANXA10 has been confirmed in three cell lines including HepG2, SMMC-7721 and Huh-7. Tumor cells are characterized by unlimited proliferation, potent migration or invasive ability, and evasion from apoptosis [25]. Therefore, it can be concluded now that ANXA10 plays a suppressor role in development or progression of hepatocellular cancer.

What is the underlying molecular mechanism of ANXA10 in HCC? ANXA10 was reported to activate the ERK/MAPK signaling pathway and promote the G1/S transition in oral cancer [11]. In colorectal cancer, ANXA10 was associated with the serrated neoplasia pathway [12]. In a study by Liu et al, ANXA10 down-regulation was shown to act synergistically with p53 mutation to cause poor prognosis in hepatocellular cancer [16]. So now there is a report to elucidate the role of ANXA10 in HCC. In this study, about

Figure 5. ANXA10 inhibited xenograft tumor growth in vivo. A. Tumor volume in ANXA10-overexpressed group was much smaller than that in control group. B. Tumor weight was lower in ANXA10-overexpressed group than that in control group. *p<0.05 was considered as statistically significant.
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2,000 downstream genes were shown to be regulated by ANXA10 in SMMC-7721 cells by gene expression array analysis. Among those 2,000 genes, 905 genes were upregulated and about 1,000 genes were downregulated. After analysis with IPA database, RhoA-ROCK signaling was highlighted. Ingenuity Pathway Analysis (IPA) is a useful tool in cancer research and has been applied extensively now [26, 27]. RhoA-ROCK signaling pathway is an important pathway in homeostasis. Abnormal activation of RhoA-ROCK signaling could lead to pulmonary artery hypertension and might be associated with epilepsy [28, 29]. In recent years, RhoA-ROCK signaling pathway was shown to promote tumor progression. For example, in osteosarcoma, miR-144 suppressed RhoA-ROCK signaling and inhibited tumor growth [30]. In ovarian cancer, activation of RhoA-ROCK signaling increased the abilities of migration and invasion of cancer cells [31]. In liver cancer, RhoA-ROCK1 signaling contributed to epithelial-mesenchymal transition and metastasis [32, 33]. In this study, IPA enrichment analysis demonstrated that the RhoA-ROCK signaling pathway was affected by ANXA10. Western blot analysis further proved that expression of RhoA and ROCK was reduced by ANXA10 in HCC cell.
Overexpression of RhoA in SMMC-7721 cells could promote cell proliferation even when ANXA10 was overexpressed, which suggested that RhoA could rescue the adverse effect of ANXA10 on cell proliferation. Therefore, we prove for the first time that ANXA10 inhibits RhoA-ROCK signaling pathway in HCC. Meanwhile, the level of Jun and MDM2 was also downregulated after ANXA10 overexpression. Jun and MDM2 are two important genes in tumorigenesis and are often increased in tumors. It is possible that Jun and MDM2 is decreased by ANXA10 in HCC.

In summary, we demonstrate for the first time that ANXA10 promotes carcinogenesis in HCC by inhibiting RhoA-ROCK signaling pathway. This study provides a new clue against HCC.

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Disclosure of conflict of interest

None.

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