Original Article

FK506-binding protein 51 could improve growth of hepatocellular carcinoma by NF-kappaB pathway

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Abstract: Objective: FK506 binding protein 51 (FKBP51) has been suggested for treating cancer and may affect the development of cancer. We want to find the function of FKBP51 in the development of hepatocellular cancer (HCC). Methods: To observe the role of FKBP51 in HCC, we chose human HCC cell line Hep3B cells to perform the experiments. And FKBP51-siRNA or FKBP51-Overexp HCC cells were generated with Hep3B cells. Then, Western blot was used to detect NF-kB levels in FKBP51-siRNA or FKBP51-Overexp Hep3B cells. Meanwhile, we generated an experimental mice model of HCC to confirm the role of FKBP51 in tumor growth in vivo. Results: The data was showed that HCC cell growth was inhibited when FKBP51 was knockdown by siRNA and enhanced when FKBP51 was overexpressed. And we also found that FKBP51-overexpressing cells showed remarkably ability of HCC migration. This result indicated that FKBP51 was correlated with cell growth and aggressiveness in HCC. Moreover, our studies in a HCC xenograft mouse model inferred that FKBP51 as an important element for the control of NF-kB activation could promote oncogenic processes by affecting carcinoma apoptosis. Similarly, evident of FKBP51 overexpression was also provided by our studies in the section from tumor samples in HCC patients. Conclusions: These data suggest that FKBP51 played a role in hepatocarcinogenesis, and we conceive that FKBP51 could be a promising biomarkers and therapeutic option for HCC cancer.

Keywords: FK506 binding protein 51, NF-kB pathway, HCC, proliferation, siRNA

Introduction

The FK506-binding proteins (FKBPs), one of the two major subfamilies of immunophilins, are the intracellular ligands of immunosuppressants FK506 and rapamycin [1-3]. FKBPs, highly conserved proteins found in many eukaryotes, are correlated with aggressiveness and therapy resistance. Accumulating studies have implicated that some FKBP members, such as FKBP38, FKBP51, FKBP52, and FKBP56, have a potential role in cancer aggressiveness and chemoresistance [4]. FKBP51 is expressed in most tissues, including liver, kidney, muscle, heart, thymus, lymph node, brain, ovary, testis, spleen, stomach, and peripheral blood leukocytes and could act as an inhibitor of steroid hormone receptors [5-7]. Interestingly, FKBP51 has been shown to be involved in cancer chemoresistance and radioresistance [2, 8-10]. A recent report demonstrated that FKBP51 could affect the sensitivity of cancer cell lines such as lymphoblastoid cell, pancreatic and breast cancer cell lines to chemotherapeutic agents [11]. In addition to its role in chemoresistance, FKBP51 was found to be overexpressed in melanoma and could regulate melanoma stemness and metastatic potential [1, 4, 10].

Indeed, FKBP51, a co-chaperone of client proteins, have been shown to be associated with pathways that could improve cell sensitivity to death in chemotherapy and radiotherapy. Recent reports reveal that FKBP51 could act as a scaffolding protein to enhance the PHPP (PH domain leucine-rich repeat protein phosphatase)-Akt interaction [4, 11]. In the process of chemotherapy, it could negatively regulate Akt phosphorylation and then induce cell death. Moreover, FKBP51 was reported to interact with the nuclear factor (NF)-kB pathway. FKBP51 could regulate NF-kB phosphorylation
and its overexpression could enhance NF-kB levels and DNA binding and then using specific FKBP51 short interfering RNA could suppress glioma cell growth [12]. Numerous publish data revealed that the relevant roles of FKBP51 in NF-kB activation were demonstrated in melanoma, glioma and leukemia [8, 12, 13].

Hepatocellular carcinoma (HCC) is one of the most common types of primary liver carcinogenesis [8, 14]. Despite the large body of studies published during the past decade that have contributed to the advance of understanding the cellular and molecular mechanisms in this field, the complex biology of HCC remains obscure and the prognosis of HCC is still poor waiting for innovative therapeutic modalities [2, 9, 10, 13, 15, 16]. In the present study, we designed to investigate the role of FKBP51 in hepatocarcinogenesis.

Materials and methods

Cell culture

Human hepatocyte cells L02 and human hepatocellular carcinoma cell lines HepG2, Hep3B, MHCC97L, Huh7 were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 pg/ml streptomycin, 100 U/ml penicillin at 37°C in a humidified incubator under 5% CO₂ in air [17].

Transfection

To generate FKBP51-overexpressing hepatocellular carcinoma cells, a pReceiver-Lv121-CMV expression vector (GeneCopoeia, Inc. China), carrying the FKBP51 gene was transfected, according to the manufacturer’s recommendations [18]. A void pReceiver-Lv121-CMV vector was also transfected to create control cells. For transfection of siRNA, 24 h before transfection, cells were grown in 12-well plates. Then, the cells were transfected with siRNA for FKBP51 (5’-ACCUAAUGCUGAGCUUAUA-3’) or with a NC siRNA (negative control siRNA, 5’-UUCUCGACGUGACGUTT-3’). After 48 h of transfection of Hep3B cells, to select for transfected cells, 500 µg/ml G418 (Biosharp, Korea) was administered for 2 weeks and until antibiotic-resistant colonies were observed [19]. Untreated Hep3B cells were used as a control, and no viable cells were observed at the same G418 concentration. The overexpression and knockdown of protein expression were determined by Western blot using anti-FKBP51 antibody and RT-PCR using specific primers for FKBP51, respectively.

Histological examination and immunohistochemical staining for FKBP51 protein

Tissue samples from liver of 4 HCC patients were embedded in paraffin wax and cut into 5-µm thick serial sections. One of slides in each tissue samples was routinely processed for hematoxylin and eosin (H-E) staining. And to determine the expression of FKBP51 protein in liver cancer tissues, the Paraffin-embedded sections were deparaffinized in xylene and rehydrated before analysis. Endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide at room temperature. After washing with PBS, the slides were blocked with 1% normal rabbit serum and incubated overnight at 4°C at 1:40 dilution in PBS of rabbit anti-FKBP51 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then incubated with secondary biotinylated goat-anti-rabbit antibody. After incubated with avidin-biotin complex for 30 min at room temperature, the slides were performed with diaminobenzidine and then counterstained with hematoxylin. Then the IOD (integrated optical density) of stained sections was measured with computerized image processing [20].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays

Tumor tissues resected from mouse are used for TUNEL assays. Sections (4 mm) from formalin fixed, paraffin-embedded tumors were deparaffinized and rehydrated using xylene and ethanol, respectively. The slides were rinsed twice with PBS and treated for 30 min at 37°C with proteinase K (15 mg/ml in 10 mM Tris/HCl, pH 7.4-8.0). Endogenous peroxidases were blocked using 3% hydrogen peroxide in methanol at 20°C for 20 min. The tissue sections were then analyzed with an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) in accordance with the manufacturer’s instructions. The reaction was visualized using light microscope.

Cell proliferation assay

Cell proliferation was measured using a Cell Counting Chamber. Cells were seeded in 24-well plate (1×10⁴/well). After a 2-day culture,
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Trypan blue (0.8 mM in PBS) was added in medium to stain dead cells. And the cells were collected and loaded onto a Burker’s counting chamber and then counted under a microscope. The growth rates of cells were determined using WST-8 assay with Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), as described in the manufacturer’s manual [21].

Soft agar colony formation assays and migrative assay

The soft agar colony formation assay was performed in 12-well plates. A bottom support layer (0.6%) of a solution containing RPMI-1640 medium, 2.4% of Noble Agar (Difco) was poured first; after solidifying, this was filled by a top layer containing the same solution and 2×10⁴ cells. Then the plates were placed in the incubator and after 14 days, colonies were counted and photographed.

Cell migration assay 4×10⁴ cells were suspended in 200 μl serum-free DMEM medium and seeded into the upper chamber of each insert. Then, 500 μL of DMEM containing 10% FBS was added to a 24-well plate. After incubation at 37°C (Ho8910 12-14 h; Ho8910-pm12-14 h; Skov3 12-14 h), the cells that migrated were fixed and stained for 30 min in a 0.1% Crystal Violet solution in PBS.

Western blotting

Mouse anti-rat monoclonal antibodies directed against IKKA, P50, and TGF-β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Also rabbit anti-β-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology. The cells were collected and total protein lysates were prepared by suspending cells in M2 buffer (20 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). Then immunoblotting was per-
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formed, equal amounts of cell lysate were resolved by 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. Proteins were visualized with chemiluminescence’s luminol reagents (Santa Cruz Biotechnology) [22].

Animal studies

After the approval of the local institutional animal research committee, nude mice (6-8 weeks old) were bought from the animal breeding facility of Sun-yet University (Guangzhou, Guangdong, China) and maintained under pathogen-free environment in the animal facility and animal studies were performed. Hep3B-overexpression and Hep3B-knockdown cells were harvested and washed twice with PBS. Aliquots of 1×10⁶ cells were suspended in 50 µl of PBS mixed with 50 µl of Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously at right and left of nude mice. Tumors were weighted using and measured using a caliper, and tumors were calculated using the following formula: tumor volume = length × width²/2 [23].

Statistical analysis

Data were presented as the mean ± SD error of the mean. Student’s t test was used for comparison among different groups. The correlation of FKBP51 expression with various clinicopathologic parameters were calculated with χ² test. The difference in tumor growth rate between the two groups of nude mice was determined by repeated-measures analysis of variance. P < 0.05 was considered statistically significant.

Results

FKBP51 is dramatically increased in samples of HCC patients and human hepatocellular carcinoma cell lines

We studied human hepatocellular carcinoma cell lines in order to found some biomolecular...
marker relative to tumorigenesis and proliferation. And we found that the levels of FKBP51 protein were increased in four HCC cell lines compared with that of normal liver cell LO2 (Figure 1A). Then the sections taken from four HCC patients were collected and stained with anti-FKBP51 antibody. Figure 1B shows the cytoplasm of HCC cells in the liver section was stained brown and the observation that carcinoma cells around vessels in liver tumors were remarkably staining suggested FKBP51 was increased in liver cancer tissues of HCC patients and these stained HCC cells may have capability of proliferation and migration. To observe the role of FKBP51 in HCC, we chose human HCC cell line Hep3B cells to perform the following experiments.

**FKBP51 positively regulate proliferation of carcinoma cell**

We generated FKBP51-siRNA or FKBP51-Overexp HCC cells using a p3XFLAG-CMV-14 expression vector. The mRNA and protein levels of FKBP51 in FKBP51-siRNA group or FKBP51-Overexp Hep3B cells were measured by RT-PCR and Western blotting (Figure 2A and 2B). The data suggested that FKBP51-siRNA and FKBP51-Overexp Hep3B cells were established. We performed WST-8 assay to investigate cell viability/proliferation. As shown in Figure 3A, higher increase in cell viability/proliferation was observed in FKBP51-Overexp Hep3B cell compared with the other two cells. And FKBP51-Overexp cells showed an increased capability to promote an anchorage-independent growth using soft agar colony formation assays. We observed that the colony number and size were dramatically increased in FKBP51-Overexp cells, suggesting FKBP51 remarkably stimulated soft agar growth (Figure 3B). Moreover, Figure 3C showed that the number of HCC cells that migrated through transwell membrane and then were stained was strikingly increased and this implied the ability of HCC migration was enhanced by FKBP51-overexpression. A contrary observation was made in FKBP51-silenced Hep3B cell.

**FKBP51 enhance NF-kB signaling in FKBP51-overexpression HCC cell**

Recently, several evidence have been clarified that the large immunophilin FKBP51 is an essential factor for NF-kB activation in neoplastic diseases, such as melanoma, glioma [24-26]. Thus, we investigated whether the FKBP51 played a relevant role in NF-kB activation in hepatocellular carcinoma cells. To verify this hypothesis, Western blot was used to detect NF-kB levels in FKBP51-siRNA or FKBP51-Overexp Hep3B cells. Our studies found that FKBP51 overexpression enhanced IKKα levels, and levels of P65 in the nucleus. As expected, the levels of IKKα and P65 were also suppressed by the FKBP51 siRNA (Figure 4A). And the levels of IKKα and P65 expression were significantly increased or reduced in FKBP51-Overexp or FKBP51-siRNA Hep3B cells compared with normal Hep3B cells (Figure 4B).

Previous studies found that MMP2, an enzyme which degraded extracellular matrix and basement membrane, have long been associated with tumor invasion and metastasis [27-29]. We also found the similar result of MMP2 expression in FKBP51-Overexp or FKBP51-siRNA Hep3B cells, which was consistent with the result of transwell assay. The data indicated that FKBP51 may be involved in tumor invasion and metastasis.

**FKBP51 promote tumor growth in HCC mouse model**

To confirm the role of FKBP51 in tumor growth in vivo, we generated an experimental mice
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A

Figure 5. A. Tumor volume was measured at the 30 day after the treatment (left panel). A representative picture of tumor growth in nude mice treated with different groups (right panel). B. Tumor weight was measured at the 30th day after the treatment (left panel). Image of the subcutaneous tumors resected in the 30th day after the treatment (right panel).

Discussion

Since the identification of the human FKBP members, including 16 proteins ranged from 12 to 135 kDa, a number of experimental evidences have been accumulated that this immunophilins played an important role in tumorigenesis. Among the human FKBP family, FK506 binding protein (FKBP51), a 51 kDa peptidylprolyl isomerase, was and associated with shock protein 90 (HSP90) and can be inhibited by macrolide immunosuppressants FK506 and Rapamycin. In glioma, FKBP51 was reported to be correlated with glioma cell growth [12]. Then recent findings suggested that FKBP51 was overexpressed in melanoma and promoted aggressiveness and therapy resistance [1]. When FKBP51 was overexpressed, glioma cell growth was promoted and vice versa. In our study, the data was showed that HCC cell growth was inhibited when FKBP51 was knockdown by siRNA and enhanced when FKBP51 was overexpressed. And we also found that FKBP51-Overexp cells showed remarkably ability of HCC migration.

model of HCC. The FKBP51-Overexp or FKBP51-siRNA Hep3B cells were injected to animals. Tumor volume in FKBP51-Overexp group cells-generated tumors was significantly higher than that in tumors derived from normal cells. And the tumor formed by FKBP51-siRNA Hep3B cells was slowly grown than that formed by control cells. At the end of observation, the mice were sacrificed and a similar observation of tumor weight was made in FKBP51-Overexp group, FKBP51-siRNA group and control group (Figure 5).

In addition, the tumor biopsies were stained with FKBP51 antibody and then analyzed using TUNEL assays. Immunohistochemistry analysis and TUNEL assay revealed that FKBP51 expressed abundantly in tumor formed by FKBP51-Overexp cells (Figure 6A), and apoptotic cells were less detected in FKBP51-Overexp group tumor (Figure 6B). Furthermore, there were high levels of apoptosis in FKBP51-siRNA group tumor. These results demonstrate that FKBP51 play an important role in tumor growth in vivo.
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Therefore, the results indicated that FKBP51 was correlated with cell growth and aggressiveness in HCC, similarly with investigations in melanoma and glioma.

In addition, we observed that the levels of IKKα and P65 expression were significantly increased in FKBP51-Overexp HCC cells. A contrary observation was made in FKBP51-siRNA HCC cells. It seemed that FKBP51 could enhance NF-κB signaling in HCC cell by regulating the level of IKKα. Jiang et al. reported that FKBP51 overexpression in glioma could enhanced levels of IkBa and regulated NF-κB phosphorylation [12]. Then, Romano et al. further demonstrated that FKBP51 played a crucial role in the control of apoptosis of irradiated melanoma cells by regulating the activation of NF-κB, and positively regulated melanoma stemness and metastatic potential [10]. Berrant NF-κB activation pathways were critical for the regulation of apoptosis and have been involved in preneoplastic and neoplastic diseases, and favored tumor invasion [26]. Moreover, our studies in a HCC xenograft mouse model inferred that FKBP51 was as an important element for the control of NF-κB activation in oncogenic processes.

Recently, numerous articles reported that NF-kB regulated expression of multitudes of genes that contributed to regulation of inflammation, apoptosis, tumorigenesis, and autoimmune diseases. And altered activation of NF-kB signaling was enhanced in human cancers [4]. Thus, it was a promising strategy of inhibit NF-kB to against cancer. In the last decade, several studies put forward that FKBP51 are critical for regulation of NF-kB activation in human neoplasia [30, 31]. Similarly, evident of FKBP51 overexpression was also provided by our studies in the section from tumor samples in HCC patients. Then, these data suggest that FKBP51 could be a promising biomarkers and therapeutic option for cancer.

In summary, we identified that FKBP51 could enhance tumor growth in HCC, and our investigation suggested that FKBP51 inhibition would be a potential molecular target for HCC therapy.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of
animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Disclosure of conflict of interest

None.

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