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ORIGINAL ARTICLE

Sorafenib inhibits liver cancer growth by decreasing mTOR, AKT, and PI3K expression

Chi-zhi Zhang[1], Xiao-dong Wang[2], Hong-wei Wang[2], Yue Cai[2], Li-qin Chao[2]

[1]Institute of Hepatology, Affiliated Hospital of Hubei University of Chinese Medicine and [2]Doctoral Candidate Department of Hubei University of Chinese Medicine, Wuhan, Hubei, China

Summary

Purpose: *The purpose of this study was to determine the impact of sorafenib on PI3K/AKT/mTOR signaling pathway and to further define its mechanism for treating hepatocellular carcinoma (HCC).*

Methods: *Human SMMC-7721 hepatic carcinoma cells were treated with or without 4 μmoL/L sorafenib. SMMC-7721 cells were harvested at various time points (0-48 hrs) and assessed for changes in PI3K, mTOR, and AKT protein and mRNA levels.*

Results: *Human SMMC-7721 hepatic tumor cells exposed to sorafenib had decreased expression of PI3K/mTOR/AKT.*

Conclusion: *Sorafenib appears to inhibit hepatic tumor growth by downregulating PI3k/Akt/mTOR signaling pathway.*

Key words: *AKT, mTOR, PI3K, primary hepatic carcinoma, sorafenib*

Introduction

Sorafenib is a protein kinase inhibitor used to treat renal cell carcinoma (RCC), differentiated thyroid carcinoma (DTC), and hepatocellular carcinoma (HCC). As a way to slow down the tumor growth and progression, sorafenib blocks the Raf/MEK/ERK signaling cascade to inhibit tumor cell proliferation, promote apoptosis and achieve antitumor effect [1]. Furthermore, sorafenib inhibits tumor angiogenesis which is required for sustained tumor growth by blocking vascular endothelium growth factors (VEGF/VEGFR02 and VEGF/VEGFR-3), platelet-derived growth factor receptor-β (PDGFR-β), and the c-kit protooncogene [2]. However, there are very few clinical reports on the relationship between PI3K/AKT/mTOR signaling and HCC. Therefore, we studied the effects of sorafenib on PI3K/AKT/mTOR signaling in liver tumor cells in an effort to possibly elucidate its mechanism of action and exploit it for possible novel therapeutic applications.

Methods

Cell culture and reagents

The human SMMC-7721 hepatic tumor cell line was purchased from JRDUN Biotechnogy Co., Ltd (Shanghai, China) and Sorafenib (200 mg/pill) was purchased from German Bayer Pharmaceuticals Company. SMMC-7721 cells were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cells were fed twice a day and only those cells in the 4-8 log phases were selected for the experimental studies. The experimental treatment group was exposed to 4 μmoL/L Sorafenib for 0, 0.5, 3, 8, 25, and 48 hrs.

Westem blot

Protein samples (40 μg) were separated by SDS-PAGE, transferred to polyvinylidene membranes, and blocked. The following primary antibodies were applied: mTOR (1:1000), AKT (1:500), and PI3K (1:1000). Subsequently, the membranes were incubated with secondary antibodies (1:3000). β-actin served as internal

Correspondence to: Zhang Chizhi, MD. Institute of Hepatology, Affiliated Hospital of Hubei University of Chinese Medicine, Wuhan, Hubei, China. Tel: +86 15827648262, Fax: +86 27 88922921, E-mail: wangxiaodong1210@126.com
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Table 1. RT-PCR primer sets

Gene		*Sequences*	*Amplified product*
β-*Actin*	H-ACTIN-S (5'-3')	GTCCACCGCAAATGCTTCTA	190bp
	H-ACTIN-A (5'-3')	TGCTGTCACCTTCACCGTTC	213bp
AKT	H-AKT-S (5'-3')	TTGTCATGGAGTACGCCAACG	
	H-AKT-A (5'-3')	ACAGCCCGAAGTCTGTGATCTT	
mTOR	H-mTOR-S(5'-3')	CCAATCATTCGCATTCAGTCC	105bp
	H-mTOR-A(5'-3)	AACAAACTCATGTCCGTTGCTG	
PI3K	H-PI3K-S(5'-3')	ATGCCTGCTCTGTAGTGGTGG	165bp
	H-PI3K-A(5'-3')	CATTGAGGGAGTCGTTGTGC	

Table 2. AKT expression levels in hepatocellular carcinoma following sorafenib treatment

	Western blot		*RT-PCR*	
Time (h)	*Control (N=3)*	*Sorafenib (N=3)*	*Control (N=3)*	*Sorafenib (N=3)*
0	0.817 ± 0.018	0.796 ± 0.037	1.000 ± 0.000	1.000 ± 0.000
0.5	0.813 ± 0.024	0.422 ± 0.039**	1.092 ± 0.007	0.217 ± 0.006▲▲**
3	0.837 ± 0.021	0.304 ± 0.038**	1.039 ± 0.011	0.053 ± 0.006▲▲**
8	0.826 ± 0.029	0.296 ± 0.033**	1.012 ± 0.008	0.043 ± 0.006▲▲**
25	0.794 ± 0.032	0.649 ± 0.041**	0.995 ± 0.017	0.227 ± 0.025▲▲**
48	0.796 ± 0.027	0.632 ± 0.027**	1.029 ± 0.036	1.167 ± 0.199▲▲**

Results are shown as mean±standard deviation.
Each time point was compared with the control group (** p<0.01) and the 0 h time point (▲▲ p<0.01).

Table 3. PI3K expression levels in hepatocellular carcinoma cells following sorafenib treatment

	Western blot		*RT-PCR*	
Time (h)	*Control (N=3)*	*Sorafenib (N=3)*	*Control (N=3)*	*Sorafenib (N=3)*
0	0.844 ± 0.016	0.809 ± 0.041	1.000 ± 0.000	1.000 ± 0.000
0.5	0.852 ± 0.021	0.960 ± 0.039*	0.935 ± 0.005	0.530 ± 0.036▲▲**
3	0.830 ± 0.019	0.463 ± 0.037**	0.995 ± 0.012	0.073 ± 0.006▲▲**
8	0.828 ± 0.028	0.431 ± 0.046**	0.915 ± 0.007	0.050 ± 0.010▲▲*
25	0.827 ± 0.022	0.821 ± 0.039	0.967 ± 0.006	0.473 ± 0.032▲▲**
48	0.834 ± 0.018	0.837 ± 0.035	1.073 ± 0.014	1.487 ± 0.064▲▲**

Results are shown as mean±standard deviation.
Each time point was compared with the control group (*p<0.05,** p<0.01) and the 0 h time point (▲▲ p<0.01).

control. The bands were subjected to grayscale analysis (Quantity One and Alpha software, Guanghzhou, China).

RT-PCR

First-strand cDNA was generated using the Rever-Tra Ace qRT-PCR kit (Shanghai, China) according to the manufacturer's instructions. Primer 5.0 software was used to design the quantitative (q) RT-PCR primer sets listed below. β-actin served as internal control (Table 1).

Statistics

Data were expressed as mean±SD. An independent two-sample t-test and ANOVA were used to compare differences between the control group and the sorafenib-treated group. All statistical tests were performed using SPSS Version 9.0 software (SPSS Inc, Chicago, Ill). A p value <0.05 was considered statistically

Table 4. mTOR expression levels in hepatocellular carcinoma cells following sorafenib

	Western blot		*RT-PCR*	
Time (h)	*Control (N=3)*	*Sorafenib (N=3)*	*Control (N =3)*	*Sorafenib (N=3)*
0	0.874 ± 0.040	0.815 ± 0.034	1.000 ± 0.000	1.000 ± 0.000
0.5	0.814 ± 0.050	0.709 ± 0.041*	1.037 ± 0.004	0.213 ± 0.006**▲▲
3	0.855 ± 0.046	0.279 ± 0.045**	1.066 ± 0.007	0.023 ± 0.006**▲▲
8	0.810 ± 0.053	0.137 ± 0.055**	1.085 ± 0.005	0.020 ± 0.000**▲▲
25	0.872 ± 0.047	0.616 ± 0.038**	1.027 ± 0.010	0.203 ± 0.023**▲▲
48	0.860 ± 0.038	0.736 ± 0.040*	1.011 ± 0.037	1.770 ± 0.137**▲▲

Results are shown as mean±standard deviation.
Each time point was compared with the control group (*p<0.05,** p<0.01) and the 0 h time point (▲▲ p<0.01).

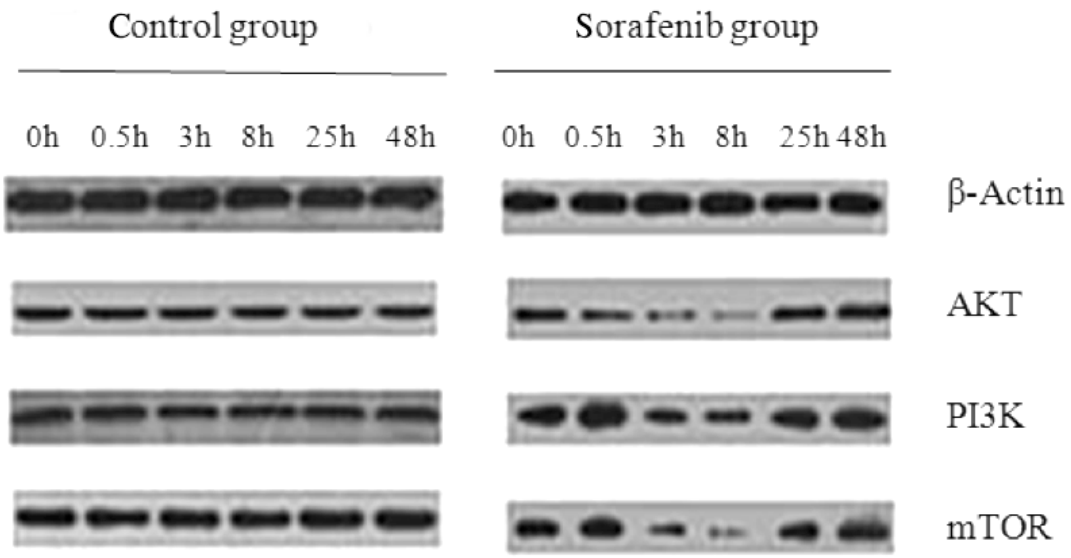

Figure 1. Control and sorafenib groups' signal pathway protein imprinting at 0, 0.5, 3, 8, 25 and 48 hours.

significant.

Results

Sorafenib downregulates AKT protein and mRNA levels in hepatocellular carcinoma

Exposing human SMMC-7721 HCC cells to 4 μmoL/L sorafenib for 0.5, 3, 8, 25 and 48 hrs significantly decreased AKT protein expression compared to 0 hours (p<0.01). These results were further confirmed by RT-PCR (p<0.01) (Table 2 and Figure 1).

Sorafenib downregulates PI3K in hepatocellular carcinoma

As shown in Table 3 and Figure 1, a 3 and 8-h exposure to sorafenib significantly decreased PI3K protein expression compared to 0 h (p<0.01). These results were further confirmed by RT-PCR.

Sorafenib downregulates mTOR expression levels in hepatocellular carcinoma

Sorafenib significantly downregulated mTOR protein levels beginning at 0.5 h (p<0.05) and continued to at 3, 8, and 25 hrs (p<0.01) post-exposure. mTOR protein appeared to recover at 48 hrs (p>0.05). RT-PCR analysis showed the same trend of mTOR gene expression after sorafenib expsosure (p<0.01) (Table 4 and Figure 1).

Discussion

Primary HCC is a highly malignant tumor and the third leading cause of cancer-related deaths in humans [3]. The formation, invasion, and metasta-

sis of liver cancer is quite complex and involves a variety of gene mutations and signaling pathway [4-8]. In this report, we demonstrated that sorafenib, a protein kinase inhibitor, regulates the PI3K/AKT/mTOR signaling cascade in human SMMC-771 hepatic tumor cells by downregulating the expression of mTOR, AKT and PI3K .

PI3K is an intracellular protein kinase that plays a role in cell proliferation and differentiation, apoptosis, glucose transport, and tumor development by activating Akt [9-11]. mTOR is another downstream mediator of P13K/Akt signal that controls cell growth and proliferation by regulating the cell cycle at G1 phase. When mTOR is mutated or overexpressed, normal cell growth becomes uncontrolled resulting in cellular transformation and tumor progression [12]. The activation of PI3K can activate mTOR, leading to rapid proliferation of tumor cells, increasing cancer protein secretion, speeding up the cell cycle, and reducing the G1 phase process [13]. Cuiqin et al. found that PI3K/AKT signaling plays an important role in the growth and proliferation of liver tumors as well as the expression of adhesion molecules [14].

Sorafenib is a protein kinase inhibitor with multiple kinase targets and is effective at inhibiting tumor growth in various cancers, including HCC. Since its discovery, sorafenib is considered a first-line therapy that can prolong the survival of patients with HCC [15]. A preclinical study by Liu et al. reported that sorafenib induces apoptosis in PLC/PRF/5 and HepG2 liver cancer cells, which suggests that sorafenib would have a curative effect in mouse models of HCC [16]. Furthermore, Jing et al. and Fengsheng et al. combined sorafenib with arsenic trioxide (As2O3) and PDD and observed an inhibition of HepG2 in a synergistic manner [17,18].

An imbalance in the PI3K/AKT/mTOR signaling cascade not only promotes tumor development, invasion and metastasis, but also plays a role in the tumor's ability to evade the host's immune system. Therefore, designing drugs that regulate PI3K/AKT/mTOR signals will have a significant impact on the prevention and eradication of liver cancer.

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