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Functional analysis of α1,3/4-fucosyltransferase VI in human hepatocellular carcinoma cells

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ABSTRACT

The α1,3/4-fucosyltransferases (FUT) subfamily are key enzymes in cell surface antigen synthesis during various biological processes. A novel role of FUTs in tumorigenesis has been discovered recently, however, the underlying mechanism remains largely unknown. Here, we characterized FUT6, a member of α1,3/4-FUT subfamily, in human hepatocellular carcinoma (HCC). In HCC tissues, the expression levels of *FUT6* and its catalytic product SLe^x were significantly up-regulated. Overexpression of *FUT6* in HCC cells enhanced S-phase cell population, promoted cell growth and colony formation ability. Moreover, subcutaneously injection of FUT6-overexpressing cells in nude mice promoted cell growth *in vivo*. In addition, elevating FUT6 expression markedly induced intracellular Akt phosphorylation, and suppressed the expression of the cyclin-dependent kinases inhibitor p21. Bath application of the PI3K inhibitor blocked FUT6-induced Akt phosphorylation, p21 suppression and cell proliferation. Our results suggest that FUT6 plays an important role in HCC growth by regulating the PI3K/Akt signaling pathway.

1. Introduction

Glycosylation is a common post-translational regulatory event during eukaryotic gene expression. However, increasing evidence suggests that aberrant glycosylation initially induced by oncogenic transformation contributes to tumor invasion and metastasis [1]. As a hallmark of cancer, aberrant glycosylation is associated with differential expressions of enzymes mainly including glycosyltransferases [2]. Fucosyltransferase (FUT) protein family is involved in the synthesis of cell surface antigen through catalyzing the transfer of GDP-Fucose to the *N*-acetylglucosamine residue of glycoproteins. FUTs have been shown to be involved in various biological processes, including cell adhesion, lymphocyte homing, embryo-fetal development and tumor progression [3,4].

The FUTs family is divided into four subfamilies, α1,2-, α1,3/4-, α1,6-, and protein O-FUT. In the α1,3/4-FUT subfamily, at least eight members have been discovered (*FUT3*, *FUT4*, *FUT5*, *FUT6*, *FUT7*, *FUT9*, *FUT10*, and *FUT11* [5–11]). Most α1,3/4-FUTs are the key enzymes in the synthesis of sialyl Lewis x (SLe^x), one kind of Lewis ligands which play important roles in cellular recognition and signal transduction through interaction with cell surface selectin receptor [12]. Particularly, the interaction between SLe^x expressing on tumor cell surface and selectin expressing on endothelial cell surface has been shown to be crucial for cancer invasion and metastasis [13]. Correlated higher expression levels of FUTs and SLe^x have been observed in metastatic tumors in pancreatic cancer and lung cancer [14,15]. Recent evidence further supported the idea that the enhanced expression of Lewis in cancer may stimulate tumor growth. While increasing *FUT4* and *FUT7* expression promoted neoplastic cell proliferation and hepatocellular carcinoma (HCC) cell growth *in vitro*, respectively, reducing *FUT3/6* expression suppressed colon carcinoma cell proliferation [16–18]. However, the underlying mechanism of FUTs-regulated cell growth remains unclear.

The major α1,3/4-FUTs activity in liver is related to FUT6 [19]. In this study, we examined the role of FUT6 in HCC. Consistent with a significant increase of FUT6 and SLe^x expression in HCC specimens, we found that FUT6 was able to promote cell growth both *in vitro* and *in vivo*. Inhibiting PI3K activity abolished FUT6-induced cell growth. Our results indicate that FUT6 acts as a tumor promoter via regulating cell growth.

2. Materials and methods

2.1. Tumor specimens

Fresh surgical specimens of HCC were obtained from Shanghai Zhongshan Hospital (Shanghai, PR China). All the samples were immediately frozen in liquid nitrogen after surgery and stored at −80 °C before further analysis.

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2.2. Quantitative real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) and then applied for reverse transcription using Reverse Transcriptase (Invitrogen). Real-time PCR analysis was carried out using the SYBR Green Supermix kit (Takara). Cycle parameters were 95 °C for 10 min hot start and 45 cycles of 95 °C for 5 s, 59 °C for 20 s and 72 °C for 10 s. Blank controls without cDNA templates were done to rule out contamination. The specificity of PCR product was confirmed by melting curve analysis and gel electrophoresis. The expression level of all genes was normalized to that of the housekeeping gene *ACTB*. Primers for *FUT6* were: forward seq., 5′-GGC TCACACCTGTAATCC-3′, reverse seq., 5′-TTAGTAGAGACGGGTTTC AC-3′, Primers for *ACTB* were: forward seq., 5′-TACCACTGGCA TCGTGATGGAC-3′, reverse seq., 5′-GATCTCCTTCTGCATCCTGTCG-3′.

2.3. Western blot

Protein samples were separated by 10% or 12% SDS–PAGE and then transferred to PVDF membranes. After blocking, the membranes were incubated with specific primary antibodies at 4 °C overnight, followed by incubating with the horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by enhanced chemiluminescence (GE healthcare). Related antibodies included: anti-SLe^x (Chemicon), anti-p21 (Millipore), anti-p27 (Cell Signaling Technology), anti-GFP (Sigma), anti-Akt and anti-phospho-Akt (Ser473) (KANG CHEN).

2.4. Cell culture and transfection

SMMC-7721 and SK-Hep1 cells were cultivated in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37 °C in 5% CO_2-humidified atmosphere. Cells with 80% confluency were transfected using the Lipofectamine reagent (Invitrogen).

2.5. Generation of stably transfected cell lines

After transfection, cells were incubated with complete medium containing G418 (Sigma) for 4 weeks. FUT6-overexpressing cell clones and mock control clones were screened and identified. To suppress PI3K/Akt signaling pathway, cells were incubated with dimethyl sulfoxide (DMSO) or the PI3K inhibitor wortmannin (1 mM) dissolved in DMSO. Cells were collected after 24 h. For generating mixed clones, three FUT6-overexpressing clones were mixed with equal proportion to generate FUT6-Mix, and two control clones to Ctl-Mix.

2.6. Flow cytometry analysis of cell cycle

For cell cycle analysis, cells were trypsinized and harvested at 80–90% confluency. After washed twice with PBS, Cells were resuspended in 0.3‰ Triton X-100 and stained by propidium iodide (10 μg/mL) in dark for 10 min at room temperature before the analysis with FACSCalibur (BD Biosciences). Cell cycle distribution was examined by the ModFit program. Results are from at least three independent experiments.

2.7. CCK8 assay for cell proliferation

Cells (1.5×10^3 cells/well) were plated in 96-well plates. CCK8 assay was used to detect cell proliferation for five consecutive days. CCK8 (Dojindo) was added to culture medium to a final concentration of 0.5 mg/mL and incubation was kept for 4 h at 37 °C. Results were obtained by measuring the absorbance at the wavelength of 490 nm.

2.8. Soft agar assay for colony formation

Cells (1.0×10^3 cells/well) were suspended in 1.3 mL 0.38% agar and plated over a 1.5 mL 0.55% agar bed in 6-well plates. The agar was prepared by DMEM containing 10% FBS. The inoculated plates were incubated at 37 °C in 5% CO_2-humidified atmosphere. Cells were cultured for about 30 days till visible colonies were observed. Colonies were identified by crystal violet staining. Results are from three independent experiments.

2.9. Tumor formation experiment in nude mice and histopathologic assessment

Four-week-old BALB/c nude mice were obtained from Shanghai Laboratory Animal Co. Ltd. (SLAC, China). Twenty mice were randomly divided into two groups. Each mouse was inoculated under the skin of lift anterior oxter with 4×10^6 cells in 0.2 mL DMEM. Tumor volume was monitored with calipers every three days once the tumors became palpable, and calculated using the formula: length × width^2/2. When average tumor volume reached 800 mm^3, mice were sacrificed for ethical reasons and tumor weights were assessed. Tumor tissues were further embedded in paraffin, cut into 5-μm-thick sections and stained with hematoxylin eosin.

2.10. Statistical analysis

In real-time PCR, the relative gene expression level normalized by *ACTB* was calculated by the formula $2^{-\Delta Ct}$, where ΔCt (critical threshold) = Ct of genes of interest − Ct of *ACTB*. For analysis of HCC specimens, fold changes of the gene expression level in tumor specimens relative to corresponding nontumorous specimens (T/N) were calculated by the $2^{-\Delta\Delta Ct}$ method and transformed to log2, where $\Delta\Delta Ct = \Delta Ct_{tumor} - \Delta Ct_{nontumorous}$. A two-tailed Student's *t*-test was used to evaluate group-level differences in our study. We considered $p < 0.05$ to be different (*) and $p < 0.01$ to be significant different (**).

3. Results

3.1. FUT6 is up-regulated in HCC

The protein expression level of SLe^x was investigated in eight paired HCC specimens (Fig. 1A). Consistent with previous reports [20,21], the protein level of SLe^x in seven tumor specimens was significantly higher than that in their surrounding non-tumorous tissues. We further investigated the expression level of *FUT6* in other 16 paired HCC specimens by quantitative real-time PCR. Thirteen out of 16 paired HCC tissues exhibited a *FUT6* expression increase in tumor specimens (Fig. 1B). This suggests that the increased expression of FUT6 during hepatocarcinogenesis may contribute to the up-regulation of SLe^x.

3.2. Transient transfection of FUT6 increases cells in S-phase

To explore the biological role of FUT6 in HCC, GFP-tagged FUT6 was transiently transfected into SK-Hep1 or SMMC-7721 cells (Fig. 2A). A dose-dependent increase of SLe^x was detected in correlation with the expression level of GFP-FUT6, indicating the biological activity of exogenously-expressed FUT6. In comparison to cells transfected with only the empty pEGFP-N2 vector, we observed a 77% and a 137% increase of the percentage of cells in S-phase in FUT6-transfected SK-Hep1 and SMMC-7721 cells, respectively (Fig. 2B). This effect of FUT6 on cell cycle regulation was further

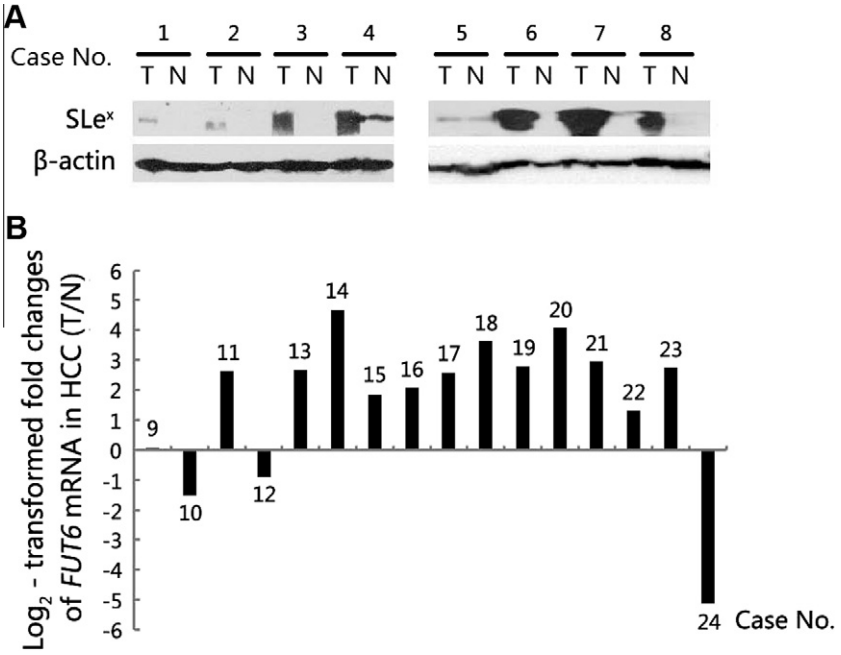

Fig. 1. The expression of SLe^x and *FUT6* in human HCC specimens. (A) The expression level of SLe^x was analyzed in HCC tissues (T) and corresponding non-tumorous tissues (N) by western blot using the anti-SLe^x antibody. β-actin was used as the internal control. (B) Log_2-transformed fold changes of *FUT6* in HCC relative to corresponding non-tumorous tissues.

supported by the finding that CDK inhibitor p21 was reduced in a FUT6 dose-dependent manner (Fig. 2C).

3.3. Stable expression of FUT6 promotes cell growth in vitro

To further investigate the role of FUT6 in cell growth, we established stable SMMC-7721 cell lines expressing GFP-FUT6 (line T9), FUT6 without tags (line F19 and F22) and empty vector (line N5 and N10), respectively. Compared to the control, line T9, F19 and F22 showed a ~3-fold increase of *FUT6* mRNA, as assessed by qRT-PCR (Fig. 3A). Consistently, the expression of SLe^x was also remarkably enhanced in FUT6-expressing stable cell lines (Fig. 3B).

Consistent with our observation on cell cycle regulation induced by transiently-transfected FUT6, FUT6 stable cell lines showed marked increases of the percentage of cells in S-phase (T9, 30.63%; F19, 32.49%; F22, 33.95%), compared to controls (N5, 19.76%; N10, 21.27%) (Fig. 3C). This result strongly suggests a role of FUT6 in promoting G0/G1-S transition of HCC cells.

To test whether FUT6 may also affect cell proliferation, we generated two mixed clones (FUT6-Mix and Ctl/control-Mix) and monitored their growth *in vitro* and *in vivo* (see Section 2). In the 5-day culture period, cells from the FUT6-Mix grew significantly faster than control cells from the Ctl-Mix, from day2 to day5 (Fig. 3D). We further examined the colony formation ability of these HCC cells in soft agar. After about 30 days incubation in soft agar, FUT6-overexpressing cells from FUT6-Mix formed 2-fold greater numbers of viable colonies than Ctl-Mix derived control cells (Fig. 3E and F), indicating that FUT6 also promotes anchorage-independent cell growth in soft agar.

3.4. Stable expression of FUT6 promotes cell growth in vivo

To get deeper insights of FUT6 functions *in vivo*, the tumorigenicity assay in BALB/c nude mice was utilized. In this assay, mixed FUT6 cells (FUT6-Mix) or control cells (Ctl-Mix) were injected subcutaneously into BALB/c nude mice (4×10^6 cells/mouse, 10 mice/group). At day 7 after cell injection, tumors were formed at a similar level in both groups. However, dramatic increases of the tumor size in the FUT6 group than that in the control group were evident since day 14 (Fig. 3G). The average tumor size in the FUT6 group at day 49 was about 799 mm^3 compared to the average size of only 419 mm^3 in the control group (Fig. 3G). In addition, the average tumor weight of the FUT6 group was approximately 66.8% heavier than that of the control group (Fig. 3H). Histology analysis with H&E staining confirmed the presence of typical phenotypes of HCC cells in both groups (Fig. 3I). Taken together, the above results suggest that FUT6 acts both *in vitro* and *in vivo* to promote cell growth.

3.5. FUT6 modulates the PI3K/Akt signaling pathway

In stable cell lines overexpressing FUT6 (T9, F19, F22), the protein level of p21, but not p27, was dramatically reduced, compared to control cell lines (N5, N10) (Fig. 4A). Together with the results from transient expression experiments (Fig. 2C), this confirms that p21 was specifically suppressed by FUT6, which may mediate the FUT6-directed cell cycle regulation.

The PI3K/Akt signaling pathway plays a critical role in cell growth, through regulating gene transcription. We thus examined the correlation of the PI3K/Akt pathway, by assessing the phosphorylation status of endogenous Akt in stable cell clones. As shown in Fig. 4A, the phosphorylation level of Akt at Ser473 was increased in all three FUT6-overexpressing cells, compared to control cells. We then asked whether the activation of the PI3K/Akt pathway mediates FUT6's effects on cell cycle. The selective inhibitor of the PI3K/Akt pathway, wortmannin, was applied to cells of FUT6-Mix and Ctl-Mix, which completely blocked the FUT6-induced G0/G1-S transition in FUT6-overexpressing cells (Fig. 4B). Consistently, wortmannin not only successfully blocked Akt phosphorylation in both cells, but also rescued p21 expression in FUT6-overexpressing cells (Fig. 4C). Our results suggest that FUT6 regulates cell cycle by modulating the PI3K/Akt signaling pathway.

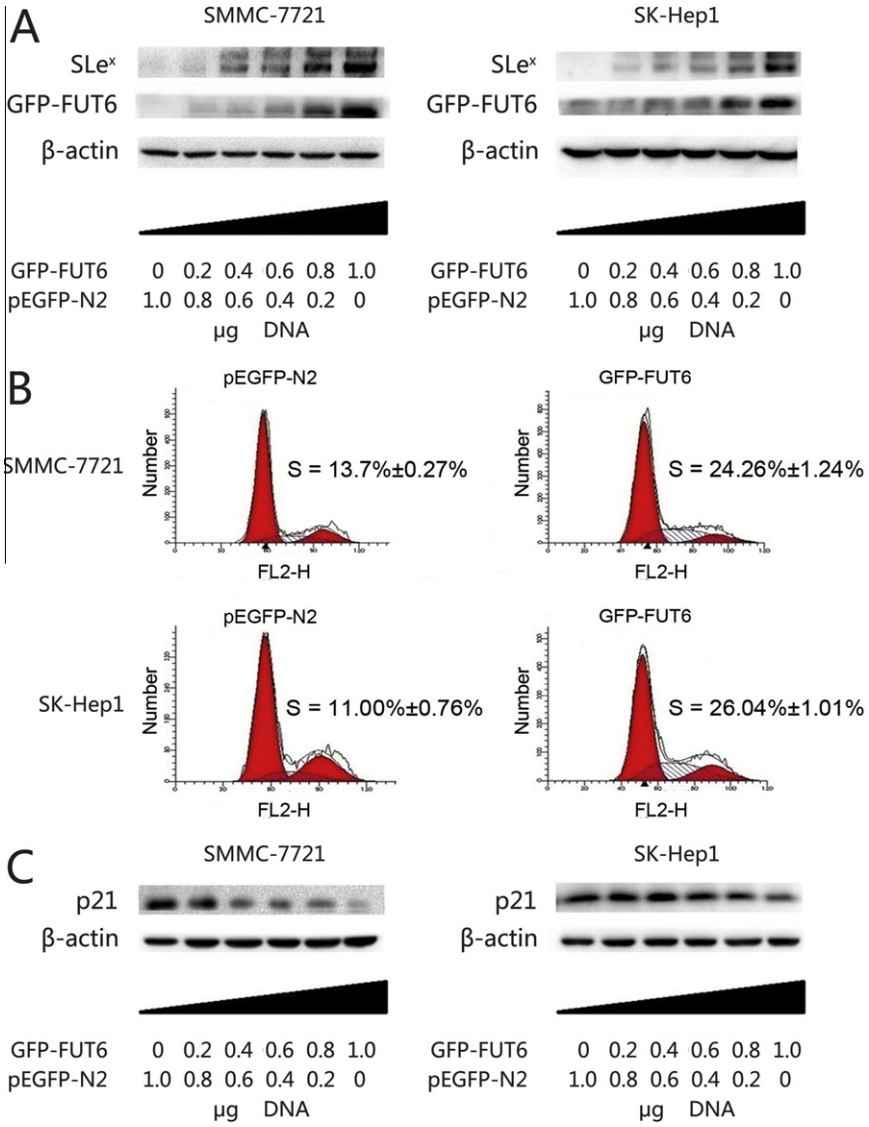

Fig. 2. Increase of the population of cells in S-phase by FUT6 overexpression. (A) Western blot analysis of the SLe^x protein level in cells transfected with indicated amounts of plasmids. β-actin was used as the internal control. (B) Cell cycle analysis with flow cytometry in cells transfected with empty vector or FUT6. Values were indicated as mean ± SD (n = 3). (C) Western blot analysis of p21 was performed in cells transfected with indicated amounts of plasmids. β-actin was used as the internal control.

4. Discussion

The correlation of the expression of α1,3/4-FUTs and SLe^x with tumor has been widely recognized in patients with colon carcinoma, gastric cancer or advanced lung cancer [22–24]. And higher expression of both FUT6 and SLe^x are also observed in colon carcinoma and pancreatic cancer [22,25]. However, the functional role of FUT6 in liver cancer progression remains unsolved.

Consistent with others' reports, we observed a marked up-regulation of FUT6 and SLe^x in HCC (Fig. 1A and B). Both cell growth ratio in dish and colony-forming ability in soft agar were dramatically increased in FUT6-overexpressing cells (Fig. 3D–F). The role of FUT6 in promoting HCC cell growth is further supported by our *in vivo* observation that cells stably expressing FUT6 exhibited stronger growth ability and therefore formed larger tumors than control cells in nude mice (Fig. 3G–H). These results strongly suggest the involvement of FUT6 in HCC.

CDK inhibitors including p21 and p27 are important negative regulators for tumor cell proliferation and growth. In HCC cells with either transient or stable overexpression of FUT6 (Figs. 2C and 4A), we detected a significant reduction of p21, but not p27, suggesting that FUT6 may release the break from CDK inhibitors and therefore accelerate HCC growth. This idea is supported by the evidence from the observation on other FUTs-mediated cell proliferation. For example, a reduction of both p21 and p27 level is detected in A431 cells with FUT4 overexpression, which correlates with an enhanced G0/G1-S transition and cell growth rate [26]. Interesting, the suppression of p27, but not p21, is observed in H7721cells and Jurkat T cells overexpressing FUT7 [17,27]. Elevated cell growth was also attributed to p27 suppression in ovarian carcinoma cells overexpressing α1,2-FUT [28]. The discrepancy on the repression of CDK inhibitors may reflect the functional differences among FUT family members.

The PI3K/Akt signaling pathway is important for cell proliferation and growth via regulating gene expression. In HCC cells with FUT6 overexpression, we observed that Akt was highly activated (Fig. 4A). In addition, inhibition of the PI3K/Akt signaling pathway with a highly specific inhibitor wortmanin reversed the effect of FUT6 overexpression on p21 suppression and cell proliferation (Fig. 4B and C). These results indicate that FUT6-promoted HCC cell proliferation is, at least in part, PI3K/Akt-dependent. Consistent with our observation, altered activity of PI3K/Akt has been found

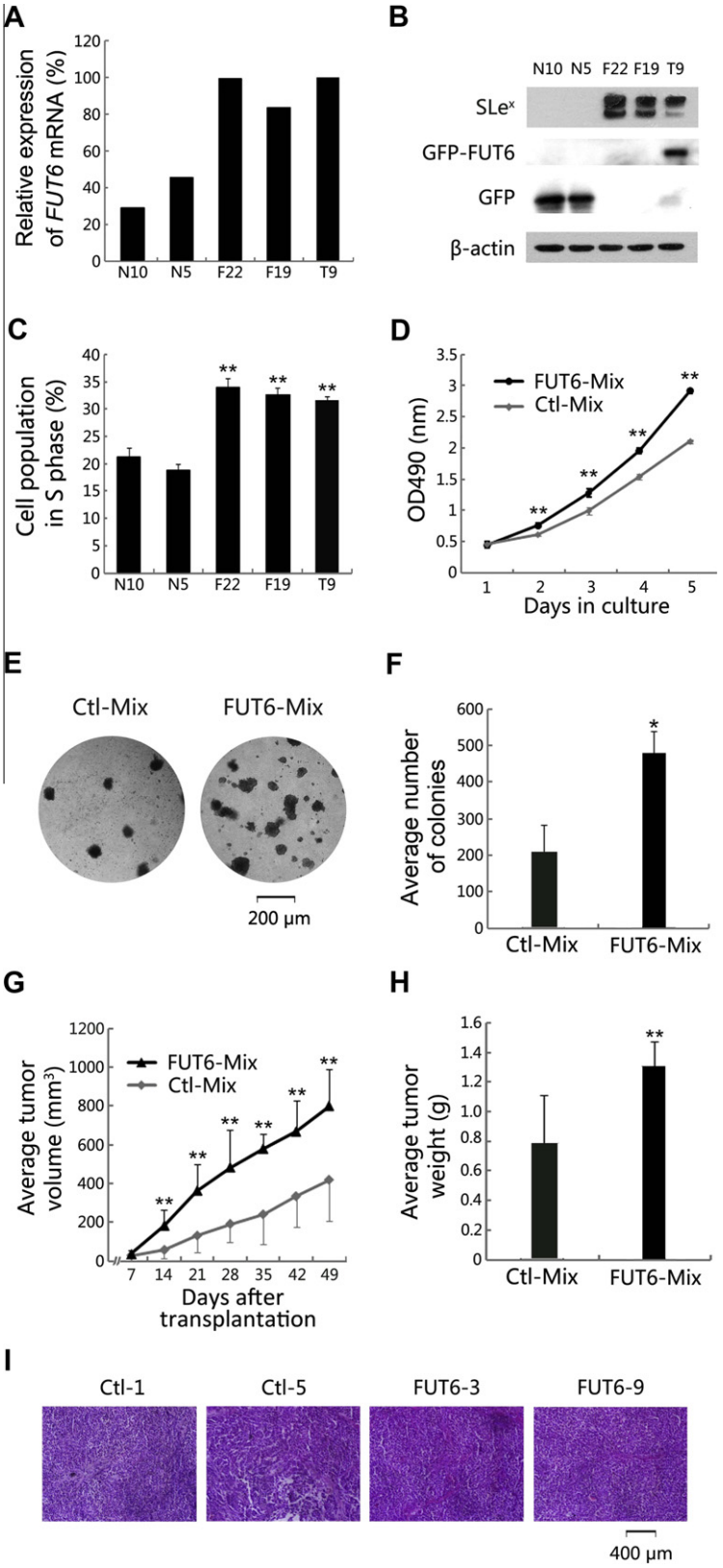

Fig. 3. Promotion of cell growth *in vitro* and *in vivo* by stable expression of FUT6 in SMMC-7721. (A) Expression analysis of *FUT6* mRNA in stable cell lines with qRT-PCR. (B) Expression analysis of SLe^x proteins in stable cell lines by western blot. (C) Flow cytometry analysis of cell cycle in stable clones. Values were indicated as mean ± SD, $n = 3$, $^{**}p < 0.01$. (D) Cell growth curves of mixed stable clones were calculated using the CCK8 kit. Values were indicated as mean ± SD, $n = 6$, $^{**}p < 0.01$. (E) Representative images of the colony formation assay in soft agar. Left, colonies formed by control cells. Right, colonies formed by clones stably expressing FUT6. Scale bar, 200 μm. (F) Statistical analysis of the numbers of viable colonies in the soft agar assay. Bar graphs represent means ± SD, $n = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$. (G) Tumor growth curves of the average tumor size recorded every three days. Values were indicated as means ± SD, $n = 10$, $^{**}p < 0.01$. (H) Statistical analysis of tumor weight. Bar graphs represent means ± SD, $n = 10$, $^{**}p < 0.01$. (I) Representative images of tumor sections derived from four representative tumors (Ctl-1, Ctl-5, FUT6-3, and FUT6-9). Scale bar, 400 μm.

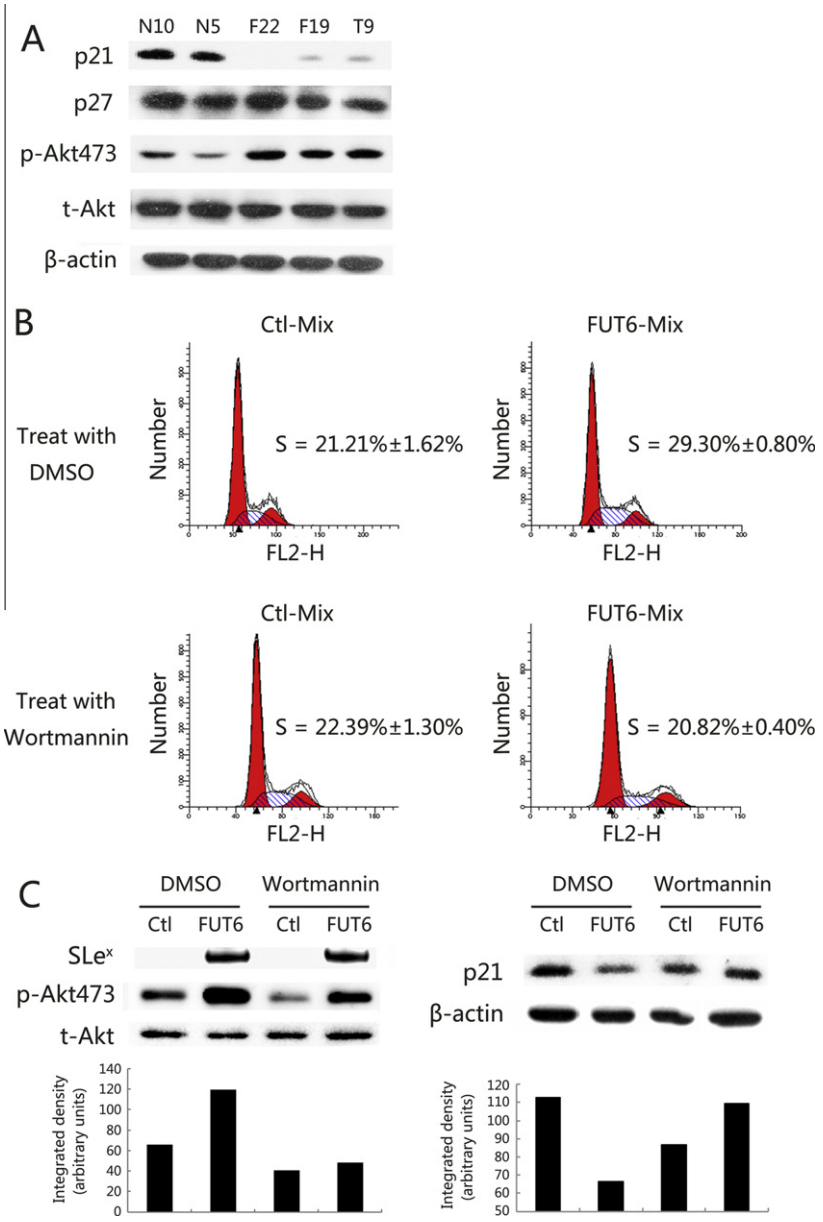

Fig. 4. Increase of Akt phosphorylation and suppression of p21 by FUT6 overexpression. (A) The expression level of p21, p27 and the phosphorylation statues of Akt were determined by Western blot using specific antibodies. β-actin was used as internal control. (B) Cell cycle profiles of FUT6 cells and control cells were analyzed after the treatment with wortmannin or DMSO. Values was indicated as means ± SD, $n = 3$, $^{**}p < 0.01$, $^{*}p < 0.05$. (C) Immunoblot analysis of Akt and p21 in cells treated with wortmannin or DMSO. Densitometric analysis was shown below the autoradiography images.

in cells expressing FUT1, FUT4, and FUT7 [26,29,30]. Interestingly, FUT4 and 7-promoted cell growth also requires the involvement of the mitogen-activated protein kinase (MAPK) pathway [26]. Further investigation is needed to understand whether the MAPK signaling pathway is also involved in FUT6-mediated HCC cell growth.

Taken together, our results confirmed that FUT6 was highly expressed in HCC and positively associated with the progression of tumor. FUT6 may play an essential role in tumor growth via modulating the PI3K/Akt signaling pathway and p21 expression. This suggests that FUT6 may serve as a promising biomarker to predict the development of HCC and a potential therapeutic target for HCC.

Acknowledgments

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